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EARLY LIFE PROGRAMMING OF PIGLETS' MICROBIOTA AND GUT HEALTH BY MATERNAL DIETARY FIBRE SUPPLEMENTATION

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Abstract

Post-weaning diarrhoea (PWD) is a widespread disease causing loss of weight and mortality of the piglets. To cure or prevent PWD, the treatment of pigs with antibiotics is frequent. The overuse of these substances led to the appearance of multi-resistant bacteria, raising public health issues. Thus, finding sustainable alternatives to antibiotics for PWD curation is of major importance. Most research focusses on the use of substances like prebiotics able to affect the microbiota of the piglets, as gut microbiota is responsible for the maturation of the intestinal immune system. Promoting a beneficial microbiota as early in life as possible is a good strategy for a better future health and a lower prevalence of PWD. Our hypothesis was that using dietary fibres (wheat bran and resistant starch) in the diet of sows would alter their microbiota and in turn affect their piglets' microbiota and future health. In addition, the ability of the two fibre sources to alter milk composition, also affecting piglets' performances and health, was tested. This hypothesis was challenged with two animal experiments.

Results indicated that wheat bran (WB) and resistant starch (RS) had the ability to alter sows' microbiota during gestation but not anymore during lactation, possibly limiting a differential microbial transfer to their offspring. These two dietary fibre slightly altered milk composition. Maternal wheat bran had the ability to increase the villus height and villus to crypt ratio in the small intestine of the progeny, while resistant starch increased the gene expression of tight junction proteins at weaning. These two fibre sources included in a high level in sows' diets did not affect their performance or their piglets', making their use in animal diets realistic.

A second objective of the thesis was to unravel whether the diet of sows could program the metabolism of piglets for later life, using them as model for human. For this, piglets were challenged with a high fat diet in order to induce low-grade inflammation and/or obesity symptoms. After 7 weeks on a high fat diet, piglets had an increased backfat thickness and higher serum cholesterol levels. The main findings are that feeding sows resistant starch increased the total sum of short-chain fatty acids (SCFA) production in the caecum and colon of their progeny, which is beneficial but did not affect the microbiota of the pigs. Moreover, maternal RS diet seemed to increase the barrier function of the colon due to a higher gene expression of tight junction proteins while the maternal effects on intestinal inflammation were contradictory for TNF-α and IFN-γ. It seems thus that the maternal diet had the ability to decrease gut permeability. However, the high fat diet did not alter the microbiota of the pigs, nor was it affected by the maternal diet.

In conclusion, using dietary fibre in sows' diet had the ability to alter their own microbiota during gestation and milk composition, but the impact on the piglet's microbiota was rather limited. It could be thus interesting to use these diets on piglets' themselves after birth to promote the establishment of beneficial bacteria. Although

effects on the microbiota were limited, the maternal diet seemed to affect some aspects of the health of their progeny in later life.

Keywords: maternal transfer, microbiota, piglet, sow, gut health, dietary fibre, wheat bran, resistant starch

Résumé

La diarrhée post-sevrage est une maladie très répandue dans les élevages porcins, causant des pertes de poids et la mortalité des porcelets. La solution la plus répandue pour pallier au problème est l'utilisation d'antibiotiques, dont l'utilisation abusive est à la source de l'apparition de souches bactériennes multi-résistantes, représentant un problème de santé publique. Trouver des alternatives durables à l'utilisation de ces antibiotiques pour traiter ou prévenir la diarrhée post-sevrage est donc d'une importance capitale. De nombreuses recherches se concentrent sur l'utilisation de substances comme des prébiotiques, qui sont capables d'impacter le microbiote intestinal des porcelets. Le microbiote étant responsable de la maturation du système immunitaire intestinal, promouvoir des bactéries bénéfiques permettrait une meilleure immunité au moment du sevrage, réduisant ainsi la fréquence des diarrhées. Notre hypothèse était qu'utiliser des fibres alimentaires (du son de blé et de l'amidon résistant) dans l'alimentation des truies pourrait impacter leur microbiote intestinal, qui pourrait à son tour affecter celui de leurs porcelets ainsi que leur santé future. De plus, l'aptitude de ces deux sources de fibres à modifier la composition du lait des truies a également été testée, étant donné que celle-ci peut affecter les performances et la santé des porcelets. Pour tester cette hypothèse, deux expérimentations animales ont été réalisées.

Les résultats montrent qu'à la fois le son de blé et l'amidon résistant peuvent modifier le microbiote intestinal des truies durant la période de gestation, mais que ces modifications ne perdurent pas pendant la lactation, ce qui peut limiter le transfert à la descendance. Les deux fibres alimentaires ont impacté la composition du lait des truies, augmentant principalement la quantité de lactose. De plus, la supplémentation avec du son de blé a résulté en de plus hautes villosités intestinales et un ratio villosités/cryptes plus élevé dans l'intestin des porcelets avant sevrage. L'amidon résistant a, quant à lui, mené à une augmentation de l'expression des gènes des protéines de jonction serrées dans l'intestin des porcelets avant sevrage. De plus, aucune de ces sources de fibres à haute dose dans l'alimentation des truies n'a détérioré les performances des truies ou porcelets, ce qui rend leur utilisation en routine réaliste.

Un deuxième objectif de cette thèse était de déterminer si l'alimentation des truies pouvait programmer le métabolisme des porcelets sur du long terme. Lors de cet essai, le porc a été utilisé comme modèle pour l'humain. L'hypothèse a été testée en soumettant les porcs à un régime alimentaire contenant une haute teneur en graisses saturées afin d'induire une inflammation chronique et/ou des symptômes liés à l'obésité. Les conclusions sont qu'alimenter les truies avec de l'amidon résistant permet d'augmenter la production totale d'acides gras volatils dans l'intestin de leur descendance sur le long terme. Cette augmentation peut être considérée comme bénéfique pour la santé même si le microbiote intestinal n'a pas été impacté par le régime maternel. De plus, le régime alimentaire maternel avec de l'amidon résistant

semble augmenter la fonction barrière du colon de la descendance, étant donné l'augmentation de l'expression des gènes codant pour les protéines de jonction serrées. L'effet maternel sur l'inflammation du colon est quant à lui contradictoire, étant donné que l'expression de TNF-α et IFN-γ ont été affectées de façon opposée. Une autre conclusion qui peut être tirée de cette expérimentation à long terme est que le challenge métabolique de 7 semaines a permis d'induire chez les porcs les premiers symptômes de l'obésité, à savoir une augmentation du cholestérol sanguin et du gras dorsal. Cependant, le microbiote intestinal des porcs n'a pas été impacté par le challenge métabolique ni par la supplémentation alimentaire des mères.

Pour résumer, l'utilisation de fibres alimentaires dans la ration des truies a permis de modifier la composition de leur lait, mais l'effet sur le microbiote intestinal de leurs porcelets est très limité. Une perspective serait l'utilisation de ces mêmes fibres dans le régime alimentaire des porcelets après la naissance en vue de promouvoir l'établissement précoce d'un microbiote intestinal bénéfique. Bien que les effets du régime alimentaire maternel sur le microbiote des porcelets soient limités, certains aspects de leur santé intestinale semblent impactés.

Mots-clés: transfert maternel, microbiote, porcelet, truie santé intestinale, fibre alimentaire, son de blé, amidon résistant.

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ADF	Acid detergent fibre
AI	Artificial insemination
APC	Antigen presenting cell
BCFA	Branched-chain fatty acids
BW	Bodyweight
CLDN	Claudin
CON	Control
CP	Crude protein
DM	Dry matter
DS	Digestible starch
EE	Ether extract
ETEC	Enterotoxigenic *E.coli*
FI	Feed intake
FOS	Fructo-oligosaccharide
GALT	Gut-associated lymphoid tissue
GE	Gross energy
GIT	Gastrointestinal tract
GLUT	Glucose transporter
HPLC	High performance liquid chromatography
Ig	Immunoglobulin
JAM	Junctional adhesion molecules
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
NDF	Neutral detergent fibre
NGS	Next generation sequencing
NK	Natural Killer cells
OCLN	Occludin
OM	Organic matter
OTU	Operational taxonomic unit
PAMPs	Pathogen associated molecule patterns
PCoA	Principal coordinate analysis
PP	Peyers' Patches
PRR	Pattern recognition receptor
PW	Post-weaning
PWD	Post-weaning diarrhoea
RS	Resistant starch

SCFA	Short-chain fatty acids
SGLT	Sodium glucose linked transporter
TJ	Tight junction
TLR	Toll-like receptor
WB	Wheat bran
WP	Work package
ZO	Zonula occludens

1

General introduction

Pig production is the first animal production worldwide, with China being the first producer and the European Union following (FAO data 2016). As the total population worldwide is continuously increasing (an increase of 32% is planned by 2050, leading to a world population of 9.8 billion according to the United Nations), so does the demand for animal products and in particular pig meat. It is thus necessary to reach high yields in a short period. In practice, several strategies are applied to improve efficiency, i.e. the selection of sows with high prolificity, an increased carcass weight and a reduced time for weaning that is very low compared to natural rearing (3-4 weeks compared to 8-12 weeks). This intensification of production in terms of abrupt early weaning was concomitant with the spread of post-weaning diarrhoea worldwide (Fairbrother et al. 2005). This disease, which is mainly caused by pathogenic *E. coli* infections (Melin et al. 2004), is characterized by lower feed intake, loss of weight, infections and mortality resulting in economic losses for the farmer. To counteract this disease, antibiotics have been widely used but their sustainability poses more and more questions nowadays. Therefore, solutions are required to prevent infections at weaning and this thesis aimed at investigating a possible strategy, by acting on the diet of the sows. In this introduction, the function and development of the gastrointestinal tract and intestinal microbiota of pigs will be described followed by a description of the weaning problems. In addition, a brief introduction to the use of the pig as a model for human disease and in particular metabolic troubles will be presented, as part of this work focussed on metabolic disorders related to ingestion of a high fat diet.

1. Gut function and maturation

The small intestine is composed of 4 layers (from the inside to the outside): the tunica mucosa, the tela mucosa, the tunica muscularis and the tunica serosa (Figure 1). Within the tunica mucosa, 3 layers are distinguished: (a) the epithelial layer, covered with exocrine (goblet cells), endocrine cells (secreting hormones) and epithelial cells (nutrients absorption); (b) the lamina propria (containing the blood and lymphatic vessels and gut-associated lymphoid tissue) and (c) the muscularis mucosa. The small intestine is very efficient in absorbing nutrients thanks to its impressive absorptive surface possible by the presence of villi and microvilli on the epithelial cells. Lieberkühn crypts, located between the villi mainly serve as a nursing for new enterocytes, having a high mitosis rate. Enterocytes will then migrate from the crypt to the villus and will replace damaged or older enterocytes cleared by apoptosis. Intestinal crypts also contain Paneth cells that produce lysozyme and defensins, protecting the intestine against pathogens. In this introduction, the emphasis lies on the small and large intestines.

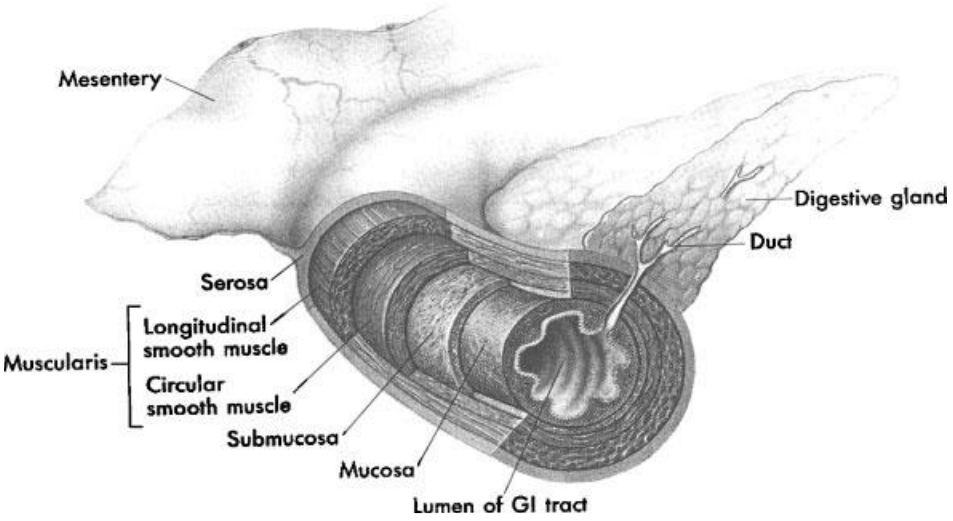


Figure 1. Structure of the gastrointestinal tract (Walthall et al. 2005).

1.1. Gut functions

1.1.1. **Digestive function & fermentation** (Sherwood et al. 2016)

The primary function of the digestive tract is to digest carbohydrates, proteins and lipids and absorb them for a release in the systemic blood flow. The first part of the digestion occurs in the stomach, where the digestion of carbohydrates and proteins begins with pepsin and salivary amylase enzymes and is continued in the duodenum. In the duodenum, the chime (pre-digested food) is mixed with pancreatic juice, containing proteases, pancreatic amylase and lipase that will convert complex proteins, carbohydrates and lipids in amino acids and short-chain peptides, disaccharides and monosaccharides, monoglycerides and free fatty acids. The digestion of lipids is complete after the release of pancreatic lipase and bile salts, while the digestion of disaccharides and small peptides needs to be terminated in the small intestine. The brush border of the epithelial layer secretes disaccharidases (lactase, maltase, sucrase and trehalase) and aminopeptidases that will allow the final digestion step of those molecules in monosaccharides (glucose, galactose, fructose) and amino acids. Glucose and galactose absorption in the enterocytes is possible by an active transport with sodium glucose linked transporters (SGLT) while fructose enters the enterocytes by a facilitated diffusion with glucose transporters (GLUT); these monosaccharides are further transported to the capillaries by GLUT2 transporter. Another way of glucose transportation is the paracellular transportation by the tight junctions between epithelial cells. The absorption of amino acids and small peptides follows the same pattern as monosaccharides, with intraepithelial peptidases able to fulfil the digestion of the di- or tripeptides (Le Huërou-Luron 2003). One exception is noteworthy for the protein passage. Indeed, the new-born piglets are equipped within the first hours of life (48h) with foetal-type enterocytes that are able to directly absorb entire proteins, like immunoglobulins from the colostrum which is necessary as the piglets are born agammaglobulinemic due to epitheliochorial placentation. The replacement of foetal-type enterocytes to adult-type enterocytes occurs within 2 days of life and is responsible for the so-called "gut closure" (Zabielski et al. 2008).

The hindgut (large intestine, comprising the caecum, colon and rectum) is important for the mineral balance as reabsorption of biliary salts, minerals, vitamins and water will happen. Some undigested carbohydrates and proteins (due to their size or their cell structure) reach the large intestine (caecum and colon) nearly intact. In the hindgut, they will be fermented by the microbiota and the end-products of their fermentation will be absorbed. This point will be discussed in another part (see section 2 "The microbiota").

1.1.2. Protective function

The gut epithelium represents a physical barrier against harmful bacteria, viruses and antigens[1] as epithelial goblet cells produce mucins that will allow the formation of a viscous gel on the surface of the epithelium (King et al. 2003). Moreover, the epithelium is sealed by proteins called tight junctions that will prevent the paracelullar transduction of macromolecules as well as bacteria; the acidity of the gastric juice and the motility of the chime are also physical obstacles for pathogens survival. Moreover, the digestive tract harbours a highly condensed and developed immune system, often referred to as the "gut associated lymphoid tissue" (GALT), composed of immune cells in the lamina propria and epithelium (like intraepithelial T lymphocytes), highly organized lymphoid follicles (of which Peyers' patches is a major representative) and small aggregates of lymphoid follicles, as summarized by King et al. (2003). Every mechanism involved in the animal protection is detailed hereunder.

1.1.2.1. Mucus layer

The mucus layer is formed after secretion of mucin glycoproteins (MUC2) and trefoil peptides (protease resistant) by goblet cells (Bourlioux et al. 2003) and has several roles s.a. the lubrication of the lumen but also a mechanical barrier against pathogens, as the tight mucus layer forms a network with a mean pore size of 100nm (Mackie et al. 2016) and exerts hydrophobic properties thanks to surfactant lipids produced by epithelial cells, preventing the passage of water-soluble toxins (Bourlioux et al. 2003). Moreover, the mucus layer serves as an habitat for commensal bacteria, representing a nutrient source (Sommer & Bäckhed 2013). Goblet cells can produce more mucins in presence of some stimuli, like toxins and bacterial infection but there is no change with aging for the piglet from day 7 to 18 of life (Brown et al. 2006) while the thickness of the mucus layer increases when going from the proximal to the distal intestine (Bourlioux et al. 2003; Mackie et al. 2016). The intestinal microbiota plays a role on the mucus layer as microbial colonization is related to higher goblet cells number and thicker mucus layer, (Sommer & Bäckhed 2013). The underlying mechanisms are so far unknown but these conclusions could be drawn from the use of germ-free animals.

1.1.2.2. Tight junctions

To connect epithelial cells (absorptive enterocytes, endocrine, Paneth and goblet cells) together and thus act as a barrier against pathogens, intracellular junctional complexes are necessary to obtain a semi-permeable dynamic and selective membrane allowing the passage of small molecules, ions and water but preventing

[1] An antigen is a molecule (often proteins) interacting with the immunoglobulin receptor of B cells (Kindt et al. 2007).

the passage of pathogens, toxins and harmful antigens (Niessen 2007). One part of this complex is formed by "tight junctions" (TJ) composed of more than 50 proteins (Ulluwishewa et al. 2011).

TJ are composed of different proteins and interface with several molecules. Tight junctions are transmembrane proteins (Figure 2), having 4 transmembrane domains and 2 extracellular loops (tetra-span tight junction, including occludin, claudin and tricelullin) or one transmembrane domain (single span, junctional adhesion molecules, abbreviated as "JAM"). Claudin proteins are involved in the tightening (CLDN 1, 3, 4, 5, 8) or the opening (CLDN2) of the paracelullar pores, while occludin (OCLN) is recognized to be involved in the regulation of intermembrane and paracelullar diffusion of small molecules (Ulluwishewa et al. 2011). Tricelullin is a protein that will tighten the closure of the membrane between 3 adjacent enterocytes, reinforcing the effect of claudins. Those tight junctions interact with plaque proteins, which are responsible for the anchoring of tight junctions into the cytoplasm of enterocytes. Important plaque proteins are zonula occludens (ZO1, 2, 3) that are composed of 3 PSD95–DlgA–ZO-1 homology (PDZ) domains that can bind to claudins, to JAM-A and to another ZO in order to form dimers. ZO-1 is thought to be very important in TJ regulation as they can interact with F-actin that are projected by the actin and myosin belt that surrounds the apical pole of the enterocytes and can thus regulate the tightening or smoothing of the TJ (Niessen 2007; Ulluwishewa et al. 2011). Moreover, ZO1- and ZO-2 have been demonstrated to determine the polymerization and extent of polymerization of claudin proteins (Umeda et al. 2006). Tight junction expression has been shown in humans to interact both with microbiota and with dietary components (Ulluwishewa et al. 2011).

1.1.2.3. The gut immune system / Gut-associated lymphoid tissue (GALT)

The immune system provides a continuous surveillance and protects the host against pathogenic infections. It is composed of primary and secondary lymphoid organs together with a collection of cells circulating in the blood and lymphatic vessels. Immunity is innate but also acquired from the contact with different pathogens; immune cells are produced and matured in the bone marrow and/or thymus and stored in secondary lymphoid organs, including the GALT. A short review of the functioning and composition of both innate and adaptive immunity is given below, before characterization of the GALT.

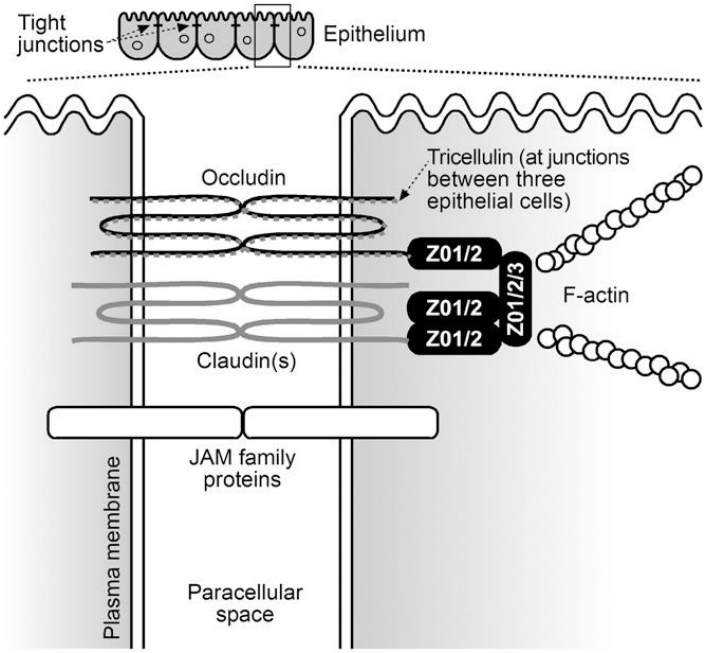


Figure 2. Schematic view of the components of the tight junctions (Ulluwishewa et al. 2011).

a. Innate immune system

The innate immune system is a very efficient defence line of the organism against pathogenic bacteria, viruses or antigens and constitutes a rapid response (Sherwood et al. 2016). This response is non-specific as it will target any pathogenic antigens and does not rely on previous contact with a specific antigen. In addition to immune cells, the innate immunity also relies on physical characteristics of the intestine, e.g. the barrier function and intestinal motility (King et al. 2003). Below is a brief summary of the main cells involved in the innate immune response. Enterocytes are able to produce cytokines after recognition of pathogen-associated molecule patterns (PAMPs) on their receptors (pattern recognition receptors, PRR including toll-like receptors, TLR) like TNFα and interferon (IFN) γ to block pathogen replication. Enterocytes have also the ability to directly produce anti-microbial peptides and pigs

do not express major histocompatibility complex (MHC) class II[2] on their enterocytes, unlike humans (Mair et al. 2014). Neutrophils represent the first defence against a pathogenic infection and have different roles, including phagocytosis of pathogens, production of anti-microbial peptides and signals for maturation, activation and attraction of macrophages and dendritic cells to the site of infection, and regulation of T-cells response (Kumar & Sharma 2010). Monocytes are differentiated in macrophages after IFNγ stimulation. Macrophages then destroy pathogens by phagocytosis and present the pathogenic antigens on MHC II together with the release of inflammatory cytokines (e.g. IL1β) that are the main communication sources between immune cells and are responsible for the recruitment of T helper cells (Mair et al. 2014). Natural killer cells have a lysis activity against pathogens and produce various cytokines including IFNγ (Mair et al. 2014). Dendritic cells are antigen presenting cells (APC) and interact with the adaptive immune system by stimulating cytotoxic and helper T cells. APC are thus essential as they will recruit the adaptive immune system; these APC include macrophages and dendritic cells. The release of cytokines related to these cells is also of major importance as they are the mediator between innate and adaptive immune responses.

b. Adaptive immune system

The adaptive immunity can be divided in two components, the humoral immunity (relying on antibodies and B cells) and the cellular immunity (mediated by T cells) (Sherwood et al. 2016). The B and T cells are produced in bone marrow while maturation occurs also there for B lymphocytes and in the thymus for T lymphocytes. The matured lymphocytes then migrate to lymphoid tissues where they establish and can proliferate (Sommer & Bäckhed 2013). Those lymphoid tissues comprise the GALT that will be described below.

The adaptive immunity relies on the detection of antigens (foreign large sized molecules that will induce a specific immune response), including dietary antigens (for which a tolerance is developed). One example of pathogenic antigen is the protein lipopolysaccharide (LPS) that is present on the cell wall of gram-negative pathogenic bacteria (King et al. 2003). Those antigens are presented by APC from the innate immunity and this presentation constitutes the first step in the activation of the adaptive immunity. Antigenic peptides are presented on MHC II.

[2] MHC are divided in two classes. MHC I is involved in the recognition of infected cells and recruits cytotoxic T cells while MHC II function is to present antigenic peptides deriving from phagocytosis to the immune system.

Humoral immunity

Specific receptors on the B cells membrane allow them to bind to a specific site of the antigen. This binding leads to the mitosis of B cells that will differentiate either in plasma cells or in memory B cells. Plasma cells will massively produce antibodies specific for the antigens that induced the mitosis. Those specific antibodies, also called immunoglobulins (Igs), are released and circulate in the blood. The recognition of the antigen by T helper lymphocytes also leads to the differentiation of B cells in plasma cells (King et al. 2003). Immunoglobulins are categorized in 5 types (IgG, IgA, IgM, IgE, IgD) even though in each type, there are millions of specific antibodies. IgG is the most abundant immunoglobulin which is present in high amounts within the blood and the colostrum of sows; IgM has a role of receptor on B cells membrane and IgA are mainly produced in the digestive tract. The effects of Igs are mainly indirect and they do not have any destructive capacity. Indeed, Igs bind to antigens, preventing them to bind to other cells, agglutinate to form insoluble complexes and act indirectly by allowing a more effective protection by activating the complement and amplifying the phagocytosis of innate immune cells (King et al. 2003; Sherwood et al. 2016). The memory B cells exist to allow a faster and long-lasting effect after a second contact with the same antigen, even years after the first attack.

Cellular immunity

In addition to threats related to the presence of bacterial antigens, viruses are able to penetrate cells and to replicate inside it. To warn the immune system of this attack or mutation, most cells can present antigenic peptides after the degradation of the viral antigens within the cells and present them on their surface thanks to MHC I. This complement allows the recognition of the presented antigen by cytotoxic (also called CD8+) T cells. Cytotoxic T cells (Tc) are thus activated, multiply and secrete factors that destroy the infected cells; these T cells are also antigen-specific and memory T cells are produced for a longer lasting protection.

In addition to Tc, two other types of T cells exist. As mentioned above, T helper (Th) cells are not killing cells but allow the modulation of other immune cells, as the proliferation of B lymphocytes and macrophages. Finally, T regulatory cells (Treg) suppress the immune response and regulate the expression of both innate and adaptive immune cells.

c. The Gut-associated lymphoid tissue (GALT)

The GALT is considered to be the largest immune organ of the porcine body, a fact that can be explained by its large surface and continuous contact with pathogenic and commensal bacteria as well as dietary antigens, for which a tolerance mechanism ("oral tolerance") has been developed (Burkey et al. 2009; Le Huërou-Luron & Ferret-Bernard 2014). The GALT is composed as any other immune organ by innate immunity (mucus layer, tight junctions, intestinal motility, phagocytic cells) and an adaptive immune system. The adaptive immune system of the gut is mainly located within the Peyer' Patches (PP), the mesenteric lymph nodes (MLN) and the lamina propria and intraepithelial lymphocytes (Le Huërou-Luron & Ferret-Bernard 2014). PP and MLN are considered as the inductive sites of the immune response, detecting pathogenic antigens and triggering the inflammatory response, while intraepithelial lymphocytes and the lamina propria are considered as the effector sites of inflammation as they will directly be able to kill the pathogens and protect the organism (Burkey et al. 2009). Peyer's Patches are present from the jejunum (as discrete PP) to the end of the ileum, where it forms a unique large PP (Everaert et al. 2017). PP surface is composed of epithelial cells without absorptive capacity and contain M cells specialized in the uptake of antigens from the intestinal lumen (containing TLR on their membrane) and thus in surveillance of the gut health. Large B-cells follicles, T cells and follicular dendritic cells are present underneath the epithelial layer (Le Huërou-Luron & Ferret-Bernard 2014; Burkey et al. 2009). M cells have the ability to transport pathogenic antigens transcellularly; these antigens will interact with the underlying APC that will present the immunogenic peptides on MHC II. T-helper cells are activated by the APC presenting pathogenic peptides and dendritic cells induce "homing receptors" on T cells that will enable them to go to the lamina propria later on. Activated T cells secrete substances (lymphokines) that induce B cells to produce antibodies. Some T and B activated cells then migrate to the lamina propria by lymphatic circulation where B cells differentiate in plasma B cells that will secrete large amounts of specific IgA, a process induced i.a. by IL-6 and TGF-β (King et al. 2003). These IgA are then released in the lumen to bind pathogenic antigens. A summary of this mechanism is shown in Figure 3.

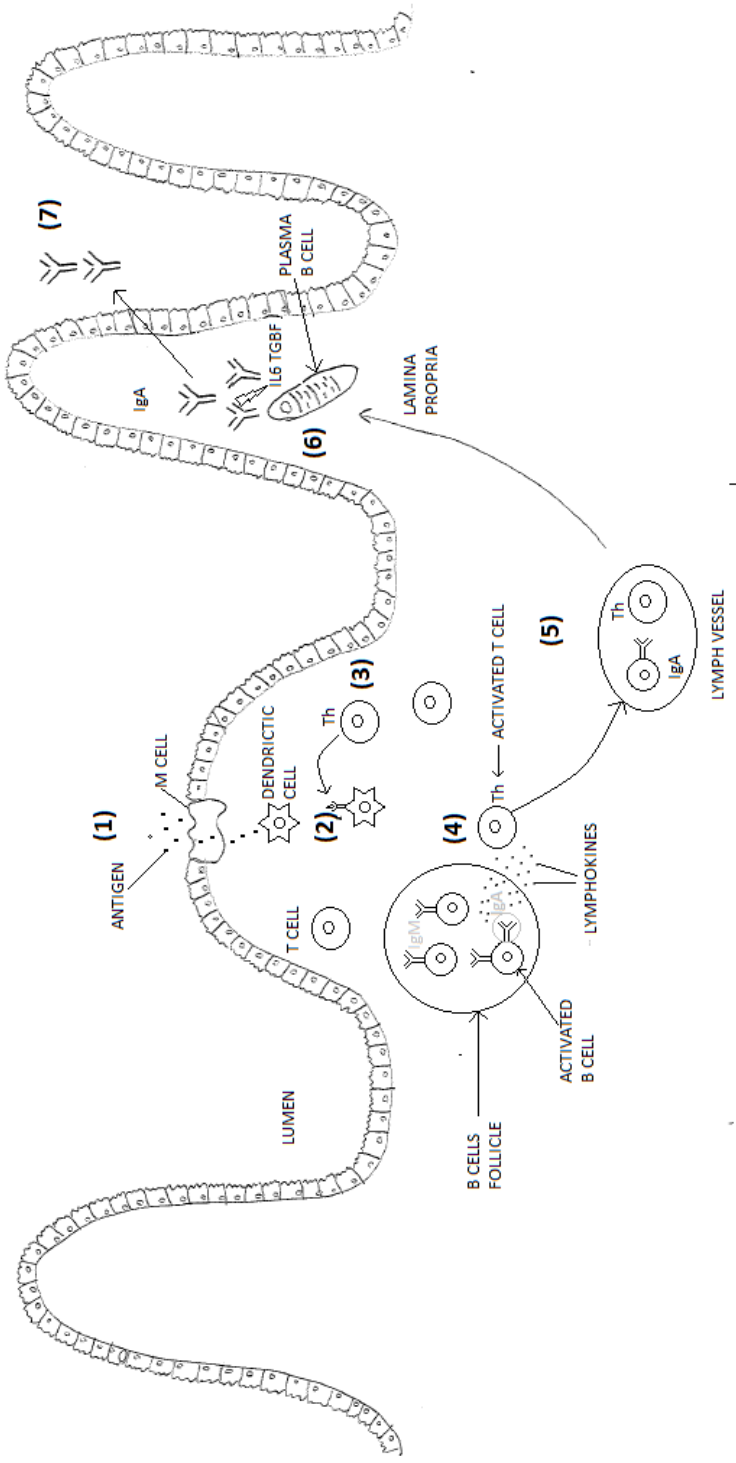


Figure 3. Mode of action of Peyer's Patches. (1) Transcytosis of pathogenic antigen by M cells; (2) phagocytosis and presenting of antigenic peptides on MHC II by dendritic cells; (3) activation of naive T lymphocytes in T helper lymphocytes and induction of "homing" receptor by the dendritic cell on T cell to direct them further to lamina propria; (4) emission of lymphokines by Thelper cells for maturation of naïve B cells that begin to produce IgA; (5) some matured T and B cells circulate in lymph vessel and thanks to "homing" receptors are directed to lamina propria; (6) differentiation of B cells in plasma cells that produce large amounts of IgA that will be secreted (7) in the lumen to bind to pathogenic antigens.

1.2. Development and maturation of the digestive tract

The gut development begins during early gestation and Buddington & Malo (1996) categorized the ontogenetic development of the gut in 5 phases. The first three phases occur during gestation (organogenesis, differentiation, growth & maturation), the fourth phase is related to ingestion and processing of milk and the switch from the neonatal intestine to a fully developed and functional adult-like intestine at weaning characterizes the fifth phase.

Everaert et al. (2017) summarized the first steps of development and differentiation of intestinal tissues and cells in a recent review. The villi present on the small intestine surface are already present from the 40th day of gestation, while the muscularis mucosae and differentiation of cells in enterocytes, goblet cells and secretory cells are achieved by the third month of gestation (Zabielski et al. 2008). It has been reported that during late gestation, the small intestine is growing faster than the body itself, probably to support the growth and need of arginine of the foetus (Sangild et al. 2000; McPherson et al. 2004; Everaert et al. 2017). The intestinal architecture is also evolving during the first days of life, as crypts depth and villi heights increase to allow a higher absorptive capacity of the intestine, together with thickening of the mucosa (Skrzypek et al. 2010). These observations also highlight the increase in absorptive area of duodenum and jejunum when piglets age, translating the maturation of the digestive and absorptive system of the piglet which is a consequence of colostrum and milk intake. Not only the architecture of the gut is modified during the first days of life, but the overall weight of the small intestine doubles during the first days of life, due to an increase of blood flow, accumulation of the colostral proteins in epithelial cells that are capable of engulfing Igs and other macromolecules, and a high mitosis rate accompanied with lower apoptosis for epithelial cells, resulting in more differentiation into goblet, immune, endocrine and epithelial cells (Zabielski et al. 2008; Le Huërou-Luron & Ferret-Bernard 2014). Epithelial cells also encounter profound changes during the first postnatal days as adult-type cells will be reached, characterized by an altered composition of transporter proteins, brush border enzymes and membrane receptors (Zabielski et al. 2008) and no ability of forming large lysosomial vacuoles like foetal-type enterocytes, leading to gut closure.

Concerning the large intestine of the piglets, the colon is not fully developed at birth and has similar functions as the small intestine (presence of villi and ability to transport amino acids) but these foetal-types colonocytes are progressively replaced by adult-type and thus loose this ability within the first two weeks after birth (Everaert et al. 2017). The replacement of foetal colonic enterocytes to mature-type enterocytes and the colonization of micro-organisms will provide the fermentative capacity that is observed in the hindgut when the pig ages.

Digestive enzymes are not fully efficient at birth. Indeed, only lactase and peptidase are already active *in utero* while glucosidase (sucrase-isomaltase and maltase-glucoamylase) activity remains low until birth (Manners & Stevens 1972; Sangild et al. 2000). Lactase expression is lower from the proximal to the distal part of the small intestine and decreases over time while sucrase-isolmaltase activity increases after birth to reach a plateau at 2 or 3 weeks (Le Huërou-Luron 2003). Aminopeptidases N and A and dipeptidyl peptidase IV are high at birth and decrease over time (Le Huërou-Luron 2003). It is reasonable to relate this lower activity over time to a lower protein content of milk compared to colostrum (Loisel et al. 2013; Krogh et al. 2017).

Due to the epitheliochorial[3] placentation of the pigs, no transfer of immunoglobulin is possible during gestation, rendering the piglet agammaglobulinemic at birth (Rooke & Bland 2002; King et al. 2003). The piglet thus relies on the intake of colostrum containing large amount of IgG and IgA and hormones within the first hours of life but also on milk during the whole lactation period as the piglet does not harbour a totally developed and mature immune system before weeks (Salmon et al. 2009; Le Huërou-Luron & Ferret-Bernard 2014). As mentioned above, this macromolecules absorption is possible by foetal-type enterocytes before the gut closure of the piglets that is complete 48h after birth (Sangild et al. 2000; Everaert et al. 2017).

Milk ingestion will trigger the absorptive and digestive functions of the small intestine of the piglets, while the microbiota colonization is involved in different functions, including the maturation of the gut-associated lymphoid tissue and fermentative ability of the large intestine (more details in 2.2).

The GALT maturation relies both on the contact with dietary antigens, occurring firstly at birth and then at weaning, and on the colonization of the gut by commensal bacteria, that will induce a tolerance of the immune system, mediated by the intervention of regulatory T cells and production of anti-inflammatory cytokines. The PP will expand within the first two weeks of life (Le Huërou-Luron & Ferret-Bernard 2014) but will not be totally mature due to the lack of IgA follicles until week 6 (Everaert et al. 2017). The effector sites of the immune response is not mature either as intraepithelial cytotoxic T cells do not populate densely the paracellular space between enterocytes until 7 weeks of age (Vega-Lopez et al. 2001) while the APC will develop in the first two weeks of life (Everaert et al. 2017). Brown et al. (2006)

[3] Diffuse epitheliochorial placentation in swine is made of 6 distinct layers separating blood from the dam and the fœtus. This means no invasion between tissues, impairing the passage of large molecules. Thus, fetal nutrition occurs by hemotrophic (diffusion or active transport) but also histotrophic (nutrition by uterine milk) nutrition (Carter & Enders 2013).

observed an increased proportion of activated Tc and activated T and B cells while ageing.

All this information together poses questions about the abrupt weaning occurring at 21 to 28 days of age in the current production systems; this point will be discussed below (see 3.1).

2. The microbiota

The gut is densely populated (10^{14} bacteria) with beneficial microbes (Isaacson & Kim 2012) that have a mutualistic relationship with the host (Sommer & Bäckhed 2013). Indeed, microorganisms populating the gut benefit from the environmental and trophic condition (lack of oxygen, temperature and substrate for growth) while the bacteria provide the host with important functions, such as the protection against pathogens and production of energy from the fermentation processes (Kamada et al. 2013; Sassone-Corsi & Raffatellu 2015).

2.1. Development of the microbiota and early colonization

The bacterial colonization of the gut begins early in life (Lawley & Walker 2013) and will be acquired by different sources, mainly maternal. Indeed, the first colonization step begins at birth when the neonate passes through the genital tract: it will ingest bacteria present in the cervix, vagina and skin (Mackie et al. 1999; Dominguez-bello et al. 2011). After birth, loads of bacteria will be ingested when suckling: bacteria present on the skin of sows will be acquired as well as bacteria and oligosaccharides (considered as probiotics) present in milk (Mackie et al. 1999; Chen et al. 2018). Moreover, bacteria from the mouth can also be transmitted by licking from the mother (Mackie et al. 1999). Another way of important bacterial transmission from the mother to the progeny is by the contact with sows' faeces, as piglets will explore the pen and ingest some (O'Doherty et al. 2017). Actually, the colonization of the progeny's gut at 3 days of age has been shown to be more related to faecal microbiota of the mother than to colonization by vaginal bacteria in humans (Sakwinska et al. 2017).

It is still under contest whether the foetus is completely sterile. In humans, bacteria have been found in the umbilical cord blood, amniotic fluid, placenta and neonate's meconium (Jiménez et al. 2005; Jiménez et al. 2008; Aagaard et al. 2014). It can be argued that the type of placentation in humans and pigs is different and this is a valid argument. However, there is evidence of trans-placental transmission of viruses (swine fever) during pregnancy as well as pathogenic bacteria (*Leptospira interrogans*) and parasites (*Schistosoma japonicum* and *Toxoplasma gondii*) from sow to foetus (Iburg et al. 2002; Soto et al. 2006; Basso et al. 2015; Girotto-soares et al. 2016) as well as evidence of pathogenic bacteria transfer in cows to the calf

foetuses (Girotto-soares et al. 2016; Abreu et al. 2017). Thus, a bacterial transfer during pregnancy from the sow to the piglets is possible despite the epitheliochorial placentation, separating the foetal blood from the maternal blood by six layers.

These bacteria will have different roles in early life, the most important being related to the development of the GALT (see 2.2). Bacteria populate the gut from the stomach to the distal colon but the bacterial composition of the different segments varies widely, as does the load of bacteria (Fava et al. 2007; Sommer & Bäckhed 2013; Yang et al. 2016). Indeed, very few bacteria are present in the stomach and duodenum due to acidic conditions and peristalsis; an increase in the terminal ileum is observed and the maximal load and diversity of bacteria is present in the caecum and colon of the animals (Lawley & Walker 2013). The relative abundance of the different phyla are also gut compartment-dependent. The jejunum has been demonstrated to be dominated by *Firmicutes* (>90%), *Proteobacteria*, *Cyanobacteria* and *Actinobacteria*; the ileum was dominated by *Firmicutes* and *Proteobacteria* (accounting for 5-40%) and caecum and colon by *Firmicutes* and *Bacteroidetes*, those two phyla accounting for more than 90% of the total microbiota (Isaacson & Kim 2012).

The microbiota of the piglets at the first day of life is similar between piglets at the phylum level (see 2.3) and then differentiates with ageing until reaching a stable community (Isaacson & Kim (2012) reported the age of 22 weeks for stabilization while Bian et al. (2016) reported 49 days). Microbiota composition is influenced by several factors including thus age, but also diet, environment, genetics and physiological state (Dominguez-bello et al. 2011). As a consequence, a high inter-individuals variation is reported in several studies (Kim et al. 2011). As microbiota of the piglets is partly acquired from the sows' faecal microbiota, several studies have been performed with the purpose of modifying the microbiota and immune competence of the piglets by changing sows' microbiota with pre- or probiotics. Even though it is known that microbiota is important for the proper development of the intestinal immune system and oral tolerance, the exact mechanisms still have to be elucidated.

2.2. Roles for host

Microbiota plays several roles for the host. The first important role is the priming of the gut immune system. Indeed, the contact with antigens will allow the proper development of the adaptive immune cells like T regulatory cells, T helper cells and plasma B cells (Kamada et al. 2013) and promote the extension of the lamina propria (Isaacson & Kim 2012) together with promoting the differentiation of immune cells like NK (Sommer & Bäckhed 2013); the exact mechanisms are not known but the interplay between microbiota colonization and GALT priming has been demonstrated with germ-free mice and piglets (Kamada et al. 2013; Everaert et al. 2017). It seems that the maturation of secondary lymphoid tissues rely on the early microbial

exposure, leading to the development of the GALT and in particular PP and MLN (Sommer & Bäckhed 2013).

A second very important role of the microbiota is the fermentation of undigested carbohydrates, including dietary fibres and resistant starch, the portion of starch that escaped enzymatic digestion due to the unavailability of this polysaccharide (due to cellulose or physical structure of starch granules). The end-products of carbohydrate fermentation are short-chain fatty acids (SCFA), namely propionic, acetic and butyric acid that constitute energy sources for the host. The end-products of protein fermentation are branched-chain fatty acids (valeric, isovaleric and isobutyric acids) and are not desirable as they will induce the formation of inflammatory compounds. From the SCFA, butyric acid is considered as the most beneficial as it constitutes the main energy source of colonocytes and can increase the expression of intestinal alkaline phosphatase, an enzyme involved in the detoxification of LPS. Other abilities of the microbiota are the physical protection against pathogens by competition for food and space, involvement in mucus production, direct production of anti-pathogenic compounds, stimulation of innate and adaptive immune responses against pathogens and production of vitamin K (Isaacson & Kim 2012; Kamada et al. 2013; Sassone-Corsi & Raffatellu 2015).

2.3. Composition of the young and adult microbiota in the hindgut

As mentioned previously, the microbiota of the neonate and the young differ from each other and differ from the complex and stable community reached in adult animals. It has been shown that within the first days of life (1-3 days of age), all piglets harbour the same microbiota type, with no influence of their breed or nursing mother, these factors driving microbiota later on (Bian et al. 2016). At the Phylum level, the microbiota of the young pig is dominated by *Firmicutes*, *Proteobacteria* and *Fusobacteria* at the first day of life, while *Bacteroidetes* begin to be dominant after 3 days of age (Bian et al. 2016), while for sows it is composed mainly of *Fimicutes*, *Bacteroidetes* and *Spirochaetes* (Larivière-Gauthier et al. 2017). A comprehensive study of the microbiota changes has been led on 10-weeks old pigs whose faeces were then collected a 5 different time points every 3 weeks (Kim et al. 2011). These authors found out that at every time point, *Firmicutes* and *Bacteroidetes* accounted for >90% of the total microbiota and that *Firmicutes* and *Spirochaetes* proportions in the microbiota increased when the pigs aged while *Bacteroidetes* decreased. Their study drew the conclusion that age and related dietary changes are the most important factors in driving microbial composition. As said previously, microbiota is not fixed and will vary in some conditions, including stress and disease (Isaacson & Kim 2012), before reaching again the climax community when the trouble ends. Microbiota of the sow or growing pig can thus be modified by the inclusion of fermentable materials in the diet; this will be discussed in section 4.4. It

is worth noting that microbiota composition changes with stress and physiological state. Indeed, studies using sows observed microbiota composition differences between the beginning and end of gestation (Larivière-Gauthier et al. 2017) and between gestation and lactation periods (Tan et al. 2016).

To determine microbiota composition of the animals, the classical method used was the culture of bacteria on specific media. However, this technique is weak as most of the bacteria present in the gut of animals are not cultivable. Thus, alternatives rose and so far, the most accurate and promising technique is next-generation sequencing (NGS). NGS relies on the amplification of 16S rRNA gene that contains hypervariable regions allowing identification of operational taxonomic units (OTU) that can be related to bacteria. This culture-independent technique has been used in this thesis.

3. Post-weaning diarrhoea

3.1. Weaning

With the intensification and the productivity reached in the current pig production systems, the age for weaning the piglets has never been so low (21-28 days today *vs* 8-12 weeks in wild pigs for the beginning of weaning process, Miller & Slade 2003). The early weaning allows a higher number of piglets to be produced per sow per year and it has been established that suckling piglets are limited for their growth because of limitation in sows' milk yield and composition (King & Pluske 2003), representing another reason for early weaning. Several stressors happen at weaning, including a brutal separation from the mother, mixing with other litters, change in the environment (sometimes transportation to another pig facility) and the conversion from milk to solid feed only (Lallès et al. 2007; Campbell et al. 2013). Moreover, weaning occurs when the animal gut functions are not totally developed (Zabielski et al. 2008), like nutrients absorption, gut immune system and secretory abilities (Lallès et al. 2004), leading to gut disorders. In wild pigs, weaning is gradual, as the sows reduce the milk they provide while the piglets begin to eat solid feed. In the pig industry, this switch from milk to solid feed is sudden rather than gradual although a creep feed is provided before weaning and forces then the immature intestine to adapt fast to a new diet (Miller & Slade 2003). Moreover, the gut immune system is not mature at 21-28 days; an example of this immaturity is the load of intraepithelial T lymphocytes that is increasing but remains low until 5 weeks for reaching adult levels at 24 weeks of age (Vega-Lopez et al. 2001). As weaning leads to the withdrawal of passive immunity provided by maternal immunoglobulins in milk, piglets are thus even more susceptible to infections.

3.2.Adverse consequences

The first consequence of abrupt weaning is a reduced feed intake (FI) within the first hours after weaning as piglets have to switch diet from palatable milk to dry solid feed. As summarized by Campbell et al. (2013), the reduced FI at weaning may have several adverse consequences on piglets' health like a lower metabolizable energy for the piglets and weight loss. Moreover, a continuous lower FI can impact the gut inflammatory status, the gut morphology and enzymes activities. One observation that has been made and summarized by several reviews (Miller & Slade 2003; Lallès et al. 2004) concerning the small intestine is the atrophy of the villi and hyperplasia of the crypts, resulting in a decreased ratio between villus and crypt in the first days post-weaning. This reduction in villus height has been mainly attributed to the lower feed intake occurring at weaning and is one of the indicators of lower intestinal integrity (Vente-Spreeuwenberg & Beynen 2003). Indeed, the altered ratio villus/crypt has been described to result in higher apoptosis of enterocytes with a lower mitosis rate, resulting in gaps in the mucosa (Zabielski et al. 2008). The decreased mucosa integrity has been associated with a higher paracellular permeability of the membrane (Boudry et al. 2004) to macromolecules s.a. toxins, pathogens and antigens to the lamina propria, where an inflammatory response can be triggered (King et al. 2003; Vente-Spreeuwenberg & Beynen 2003). Another consequence of the weaning process itself and of the lower villi (result of lower FI) is the reduction of the brush border enzymes activity (Vente-Spreeuwenberg & Beynen 2003). As summarized by Le Huërou-Luron (2003), lactase and sucrase activities decreased within the first 4 days post-weaning (PW) when piglets were weaned at 2-3 weeks of age while sucrase activity recovers within 11 days post-weaning (PW). As microbiota composition relies both on nutrients availability to the bacteria and on the physiological state of the animal, weaning induces important changes in the microbiota composition of the host, making the gut more susceptible to proliferation of pathogenic bacteria (Castillo et al. 2007). All the factors together (disturbed microbiota, lower feed intake and digestion, more exposure to pathogens, immature gut immune system and no passive protection from sows' milk), summarized in Figure 4, increase the risk of enteric infections at weaning which is often observed in the farm as piglets experience post-weaning diarrhoea (PWD). PWD is most often the result of infections of the proximal intestine with enterotoxigenic *E. coli* (Hopwood & Hampson 2003; Gresse et al. 2017) which fimbriae bind to the enterocytes and releases toxins causing secretion of water and electrolytes in the faeces. PWD together with anorexia of the animals cause a loss of weight, disease and can finally lead to mortality of the animals. These problems represent for the farmer an economic loss as the time to slaughter will also be increased and the piglets will have to be cured with medication.

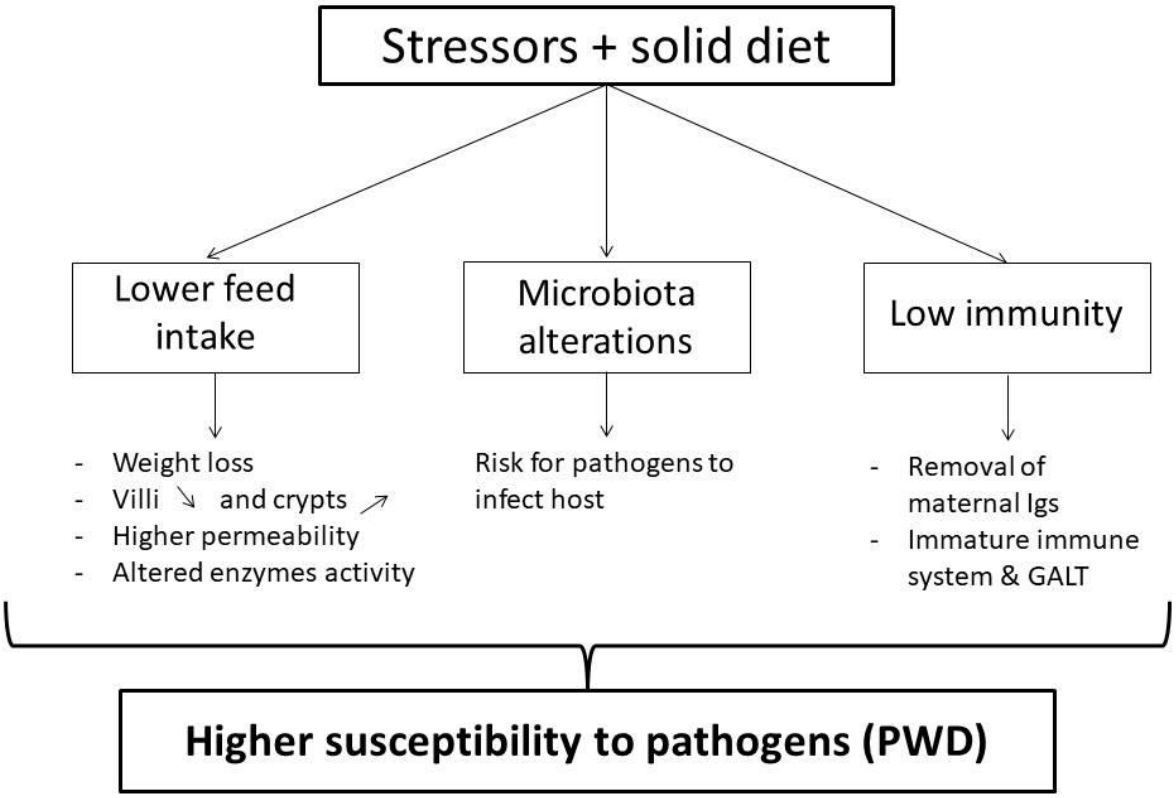


Figure 4. Main mechanisms underlying the susceptibility of piglets to PWD.

3.3.Allopathic management: antibiotics and zinc oxide

Traditionally, antibiotics have been used to cure and prevent PWD. It had been observed that antibiotics could act as growth promoters at low doses in-feed, and this observation led to the massive use of antibiotics as growth promoters all around Europe (Levy 2014). As the exposition of non-lethal doses of antibiotics to pathogens promotes the appearance of resistant genes, a global threat for animals and humans health has emerged with the rise of multi-resistant bacteria. Because of this global health issue, the European Union banned progressively the use of in-feed antibiotics as growth promoters (Heo et al. 2013). In Belgium, AMCRA objectives are a reduction of 50% of antibiotics use from 2011 to 2020. In 2016, the cumulative reduction was of 20%, from which the highest decrease is for in-feed premixes (BelVet-SAC 2017).

Thus, more sustainable alternatives to antibiotics are necessary and zinc oxide has been used for years now to prevent PWD at therapeutic doses as it has been authorized in Belgium in 2013 (BelVet-SAC 2017). As summarized by Heo et al. (2013), the use of ZnO in weaner diets increases the gene expression of microbial peptides and IGF-I and IGF-II in the small intestine and alters the gut microbiota. However, the use of ZnO is very pollutant and highlights the need for more sustainable alternatives.

3.4.Alternatives to antibiotics and zinc oxide

Several feed alternatives have been proposed and are under the focus of different studies: probiotics, prebiotics and organic acids are the most promising additives in regard to improving gut health. In this introduction, the focus will be put on prebiotics and in particular dietary fibres. Prebiotics have been firstly described in 1995 by Gibson & Roberfroid (1995). In 2004, Gibson et al. (2004) highlighted the need for a new definition of prebiotics. This definition of prebiotics includes several characteristics, including the resistance of the substance to enzymatic digestion, the fermentation by microbiota and the ability to selectively stimulate the growth and/or activity of beneficial bacteria. Thus, strategies have been developed aiming at stimulating the survival and growth of beneficial microbes within the microbiota at the expense of pathogens. In particular, bacteria producing butyrate have been targeted. In addition to changes in microbiota composition, studies using prebiotics also target inflammatory status (Leonard et al. 2012; Le Bourgot et al. 2014), gut morphology and permeability (Chen et al. 2013; Chen et al. 2014), diarrhoea score (Kim et al. 2008; Walsh et al. 2012) and performances of the pigs. Most studies focus on the introduction of these substances in the growing pigs' diets, but few focused on early nutrition, i.a. on the introduction of prebiotics directly during the suckling period (Wang et al. 2013) or weaner diets (Kim et al. 2008; Walsh et al. 2012; Hong Chen et al. 2013; Chen et al. 2014) or indirectly in sows' diets. Only studies related to sows' nutrition will be discussed here.

In this thesis, the focus was put on the introduction of fibres in sows' diet to induce microbiota, immune and morphological changes in the progeny. The hypothesis underlying this objective is that microbiota of the sow will be transmitted to the offspring. As microbiota is important in the development of the immunity, it is likely that acting on sows' microbiota will allow the modulation of the immune system. Moreover, the colostrum has a strong influence on the immunity of the piglets as it carries important quantities of passive immunity, in the forms of secretory IgA and IgG. Milk composition (protein, lactose and fat) are also important for the survival of the piglets and for shaping the gut morphology. Some studies got interested in maternal nutrition concerning one or several of these parameters. Table 1 summarizes the results of some studies concerning the maternal transfer. In addition to the studies in the table, other studies focussed in the alteration of milk composition following the inclusion of dietary fibres in sows' diet. An increased milk protein concentration was reported for sows fed alfalfa or high fibre diets (Loisel et al. 2013; Krogh et al. 2017). Loisel et al. (2013) also observed an increase in fat concentration for sows fed a high fibre diet.

Table 1. Overview of studies using maternal nutrition to impact piglets' microbiota, gut morphology and inflammation and passive immunity provided. G=gestation.

Supplementation source	Amount	Period	Piglets' age at sampling	Effects	Reference
Seaweed extract	10g/day	day 104 of gestation - weaning	26 days	Colostrum IgA increase decreased *E.coli* in colon of piglets at weaning TNFα expression increased in LPS challenged ileum No impact on gut morphology No impact on performances	Leonard *et al.* (2012)
Short-chain fructooligosaccharide	3% of the diet	day 87 of gestation - weaning	21 days	IgA in colostrum and milk day 6 increased Colostrum TGFβ1 increased Increased sows backfat thickness days 14 and 28 of lactation sIgA increased in MLN, jejunal and ileal PP Increased CD4+ T helper cells Increased piglets' plasma haptoglobin No impact on SCFA	Le Bourgot *et al.* (2014)
Seaweed derived polysaccharides	10 g/day	Day 83 of gestation - weaning	28 days	Lower sows' faecal *Enterobacteriaceae* , no impact on piglets Jejunum: higher villi and crypts Ileum: higher villi, lower crypt, higher V:C Lower expression of PEPT1, SGLT1, SGLT2 Higher expression of ileal IFN-γ, IL-1, IL-12A, TGF-β1, TNFα Lower expression of ileal IL-6, IL-8, IL-10, IL-17R	Heim *et al.* (2015)
Inulin	3% of the diet	Day 93 of gestation - 14 days lactation	10 days	Higher count of *Enterococci* in sows' faeces & piglets' caecum Lower faecal pH for sows Higher C. leptum in caecum of piglets	Paβlack *et al.* (2015)
Chitosan oligosaccharide	3% of the diet	Day 85 of gestation - day 14 of lactation	14 days	Ileum and jejunum: higher Villi and V:C	Xie *et al.* (2016)

4. Dietary fibre: wheat bran and resistant starch

As observed in Table 1, dietary fibres and prebiotics are good candidates to be introduced in maternal diet, thanks to their ability to modify sows' microbiota and milk composition. In this thesis, two sources of fibres have been envisaged.

Wheat bran (WB) was firstly used as it presents several benefits. WB is the outer layer of the wheat grain, including the cuticle, pericarp and seed coat and is a by-product of the milling industry. WB is already used in animal feed as it is a cheap ingredient and presents interesting bulking properties helping sows to cope with frustration induced by the feed restriction during gestation. WB is a source of insoluble non-starch polysaccharides and is rich in arabinoxylans (22-30%), cellulose (9-12%) and lignin (3-5%) (Kamal-Eldin et al. 2009) and is thus a good candidate to be included in the diet as it will be fermented in the hindgut (Govers et al. 1999; Bach Knudsen & Canibe 2000). WB has been mostly studied on weaned piglets and the main conclusions concern the fermentation end-products, e.g. an increased production of total SCFA, an increased proportion of acetic acid and a decreased branched chain fatty acids (BCFA) production, which is beneficial as they are related to protein fermentation and lead to the production of inflammatory compounds in the gut (Martín-Peláez et al. 2009; Molist Gasa et al. 2010; Molist et al. 2012; Nielsen et al. 2014; Iyayi & Adeola 2015). The impact of WB fed to growing pigs has been demonstrated to impact microbiota as Molist et al. (2012) observed a decreased abundance of *Bacteroidetes* in the faeces of pigs fed 4% of coarse WB, while Ivarsson et al. (2014) observed an increased abundance of *L. reuteri* (able to produce antimicrobial compounds) in the ileum and of *M. elsdensii* (a bacterium able to exclude the pathogen *Brachyspira hyodysenteriae*) in the faeces of pigs fed 14% of WB. In weaned piglets' faeces, a trend for a decreased abundance of *Enterobacteriaceae* (Molist Gasa et al. 2010) and an increase in *Bifidobacterium* (Chen et al. 2013; Yu et al. 2016) were reported when feeding them 8% or 10% of WB, respectively, together with a lower copy number of *E. coli* in the faeces (Chen et al. 2013). Moreover, Zhang et al. (2016) observed an impact of providing suckling piglets with creep feed containing 2.92% of WB as *Dorea spp.* increased in the caecum, while *L. paracasei* increased and *S. suis* decreased in their distal colon. Effects on the immune competence and permeability of the membrane of weaned piglets have also been reported when supplementing them 10% of WB in the diet (Chen et al. 2013; Chen et al. 2017). The observed effects were an increased expression of tight junction proteins (ZO-1 and OCLN) and decreased expression of pro-inflammatory compounds (e.g. IL-6, TNFα) in the ileum.

All these studies designated WB as a good candidate to be introduced in the diet of sows for the first experiment and another feed ingredient was tested during a second animal experiment. Resistant starch (pea starch) was selected based on literature and on its properties. Resistant starch (RS) is the part of starch that is able to escape enzymatic digestion in the small intestine due to chemico-physical properties and is thus fermented in the caecum of the animals (Haenen et al. 2013; Nielsen et al. 2014; Giuberti et al. 2015). Depending on the characteristics of the starch, it will be divided in 5 categories (RS1: physically inaccessible starch, RS2: native resistant starch granules, RS3: retrograded starch, RS4: starch that has been chemically modified and RS5: amylose-lipid complex starch, Giuberti et al. 2015; Yan et al. 2017). Thought to influence the microbiota and SCFA and butyrate production (Pieper et al. 2015), resistant starch is thus another good candidate for inclusion in sows' diet. Several studies showed that RS can act on microbiota, SCFA production, immune development or milk composition. The impact of the starch on these parameters will differ depending on the type of RS used. Indeed, Martinez et al. (2010) observed an effect on humans' microbiota when using RS4 but not with RS2. *In vitro* (Giuberti et al. 2013) or *in vivo* studies on growing or adult pigs using resistant starch, in the form of RS2 or RS3, determined the production of total and individual SCFA. A higher production of total SCFA, acetic acid and propionic acid inducing a lower intestinal pH were observed, which is considered as beneficial to prevent the growth of pathogens (Bird et al. 2007; Haenen et al. 2013; Nielsen et al. 2014) together with a lower production of branched-chain fatty acids and molecules produced during protein fermentation (Haenen et al. 2013; Zhou et al. 2016). In general, an increased butyrate concentration is observed, which however seems to be segment-dependent (Bird et al. 2007; Nofrarías et al. 2007; Haenen et al. 2013). The effects of resistant starch supplemented to pigs have also been related to microbial changes in the hindgut (Sun et al. 2015) Indeed, Haenen et al. (2013) observed a change of 7 bacterial groups in the caecum and 30 bacterial groups in the colon (including the beneficial bacterium *Faecalibacterium prautsznii*) when feeding adult female pigs 34% of RS3. *Bifidobacterium* and *Lactobacillus*, also considered as beneficial bacteria, seem to be impacted by supplementation of RS (Bird et al. 2007; Regmi et al. 2011). An improved integrity and health of the intestine by RS have also been reported as Zhou et al. (2016) observed an increased expression of genes involved in mucin production while Nofrarías et al. (2007) observed an increase of some goblet cell types, an increased thickness of tunica muscularis together with a lower number of apoptotic cells. Table 2 summarizes the beneficial effects of these two feed ingredients on gut health.

Table 2. Summary of the effects of wheat bran (WB) and resistant starch (RS) on several parameters of the intestinal content, mucus or faeces. #Microbiota changes comprise changes at relative abundance of beneficial genera and overall changes of microbiota (composition, diversity); * Gut health comprises effects on mucus production, tight junctions and/or inflammation of the intestine. 1: Bird *et al.* (2007) ; 2 : Chen *et al.* (2013); 3: Chen *et al.* (2015); 4: Haenen et al. (2013); 5: Ivarsson et al. (2015); 6: Iyayi & Adeola (2015); 7: Martin-Pelaez et al. (2009); 8: Molist Gasa et al. (2010); 9: Molist et al. (2012); 10: Nielsen et al. (2014); 11: Nofrarias et al. (2007) ; 12 : Regmi et al. (2011) ; 13 : Sun et al. (2015) ; 14 : Yu et al. (2016) ; 15 : Yan et al. (2017); 16 : Zhang et al. (2016) ; 17 : Zhou et al. (2016).

Parameter	Effect	WB	RS
Total SCFA	↗	✓ 6, 7, 9	✓ 1, 4, 10
Acetate concentration	↗	✓ 6, 7, 9	✓ 1, 4, 10
Propionate concentration	↗	✓ 7	✓ 1, 4, 10
Butyrate concentration	↗	✓ 5, 8	✓ 1, 4, 10, 11
BCFA & toxic compounds	↘	✓ 6, 7, 8	✓ 4, 17
Microbiota changes#	+	✓ 2, 5, 9, 14, 16	✓ 1, 4, 12, 13, 15
Gut health*	+	✓ 2, 3	✓ 11, 17

5. The pig as model for human gastrointestinal tract

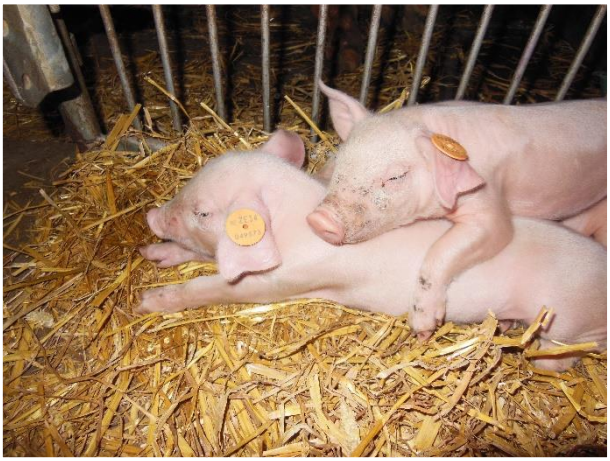
The study of the gastrointestinal tract (GIT) development and intestinal challenges for human purposes need appropriate animal models whose GIT development and function are similar with that of humans. Historically, mostly rats and mice have been used for studies because of their low cost, rapid reproduction and handling ease (Guilloteau et al. 2010a; Gonzalez et al. 2015). However, rodents may not be the most suitable model for GIT studies. Indeed, rodents gestation length and thus maturity of the new-born is different from human, they are granivorous animals, the fermentation of carbohydrates happens in the caecum rather than colon and they have a lower fermentative ability due to faster passage rate of the digesta through the intestine (Guilloteau et al. 2010a; Heinritz et al. 2013; Gonzalez et al. 2015). On the contrary, pig as a large animal model shares most of the digestive characteristics of the humans. The first common characteristic is the same omnivorous diet. Moreover, those two species share a long gestation, enabling the piglet to gain maturity for enzymes activities and gut morphology before birth; however, it is noteworthy that due to different placentation, the piglet is born agammaglobulinemic as specified above, which renders the foetal-type enterocytes quite different from human as a closure of the mucosa will happen within the first two days of life for piglets. Other advantages of using the pig model include the same gut characteristics (same transit time, mainly colonic fermentation, same digestive and absorptive processes) and a microbiota composition that is more similar to human than microbiota of rodents compared to humans.

Concerning microbiota, the dominating phyla are the same (*Firmicutes* and *Bacteroidetes*) for pigs and humans as well as the relative abundance of *Lactobacillus*, a genus for which the abundance differs for rodents (Heinritz et al., 2013). However, *Bifidobacterium* (~4% in human colon) are present but to a lower extend in the pigs' colon, even though studies mostly focused on the neonatal or un-weaned piglet, for which *Bifidobacterium* is very poorly represented (<1% of *Bifidobacterium*) (Heinritz et al. 2013). Even though microbiota of the pigs and humans are not exactly the same, the physiological and anatomical similarities (responses to diseased state, colonic fermentation) make the pig a good animal model even for microbiota studies, which is supported by the successful transplantation of human faecal microbiota in gnotobiotic pigs, which was not efficient enough in rodents for which *Bifidobacterium* was not able to colonize the gut (Heinritz et al. 2013).

Pigs are becoming extensively used models for GIT disorders, including necrotizing enterocolitis or troubles related to obesity (adiposity and dyslipidaemia), as pigs and humans have the same body fat distribution and adipose cells size, even though the major site for lipogenesis is different (adipose tissue for the pig, liver for human; Heinritz et al. 2013). Despite the high similarities between

pigs and humans, it is important to note that all breeds are not suitable for studies concerning the development of type 2 diabetes syndromes and in particular the development of insulin resistance and obesity. Indeed, conventional pigs have been selected for leanness, conducting to low body fat deposition (Renner et al. 2016); one solution is the use of rustic breeds or minipigs that have the ability to deposit more intramuscular and subcutaneous fat (Torres-Rovira et al. 2012; Renner et al. 2016). In addition, pigs don't develop insulin resistance characterized by destruction of beta-cells in the pancreas; solutions proposed by Renner et al. (2016) are the use of transgenic pigs or of substances able to destroy insulin-producing beta-cells.

Pigs are also used as model for preterm delivery, food deprivation during pregnancy and infant early nutrition (Thymann et al. 2009; Ferenc et al. 2014; Mudd & Dilger 2017). However, studies concerning the use of pigs around birth and weaning need to be taken carefully as weaning is more brutal for pigs and as piglets are not as mature as human neonates at birth. To conclude, pig and piglets are valuable tools for research oriented to human health, even though their cost and difficult handling must be taken into account.



2

Objectives, methods and articles related to the thesis

1. Objectives

The aim of the thesis was to unravel the potential of two dietary fibre sources (WB and RS) included in sows' diet to impact their microbiota, milk composition and piglets' health. Indeed, we investigated if sows' microbiota and milk composition were affected by their dietary treatment and if this might in turn have impacted the microbiota and gut health-related parameters of their piglets by different mechanisms (Figure 5). Firstly, as faeces of the sows are in direct contact with piglets, a first microbial exposure and colonization occurs with sows' microbiota; thus, modifying sows' microbiota by dietary fibre nutrition, could differentially affect their piglets' microbiota (1). A possible transfer of microbiota already during gestation was also investigated. Microbiota in turn, by fermenting dietary compounds, produces SCFA (2); these SCFA have direct positive effects on the energy balance of the piglets and can interact with the immune system (3). On the other hand, an altered milk composition might also impact piglets' gut health related parameters. Firstly, milk nutrients load and yield can impact the growth and survival of the piglets (4) and their gut architecture (5). Then, colostrum and milk provide passive immunity to the piglets by the transfer of immunoglobulins (6). Finally, milk composition can impact gut morphology and indirectly affect piglets' microbiota (7). Thus, we hypothesized that acting on both sows' microbiota and milk composition by the use of in-feed dietary fibres could promote a beneficial microbiota for the piglets early in life and improve their intestinal health. This hypothesis was tested with two animal experiments. Additionally, a long-term study with a metabolic challenge was performed. Indeed, during the second animal experiment, the pig was used as an animal model for human using RS in sows' diet. The hypothesis was that feeding sows with RS would alter the physiological responses (cholesterol production, backfat deposition, gene expression) and microbiota of their progeny submitted to a high-fat challenge in the later life.

It is noteworthy that the piglets might have been metabolically programmed during foetal development and that changes in sows' metabolism could have altered their milk composition. This hypothesis was however not investigated in the current PhD thesis, but is considered in future research.

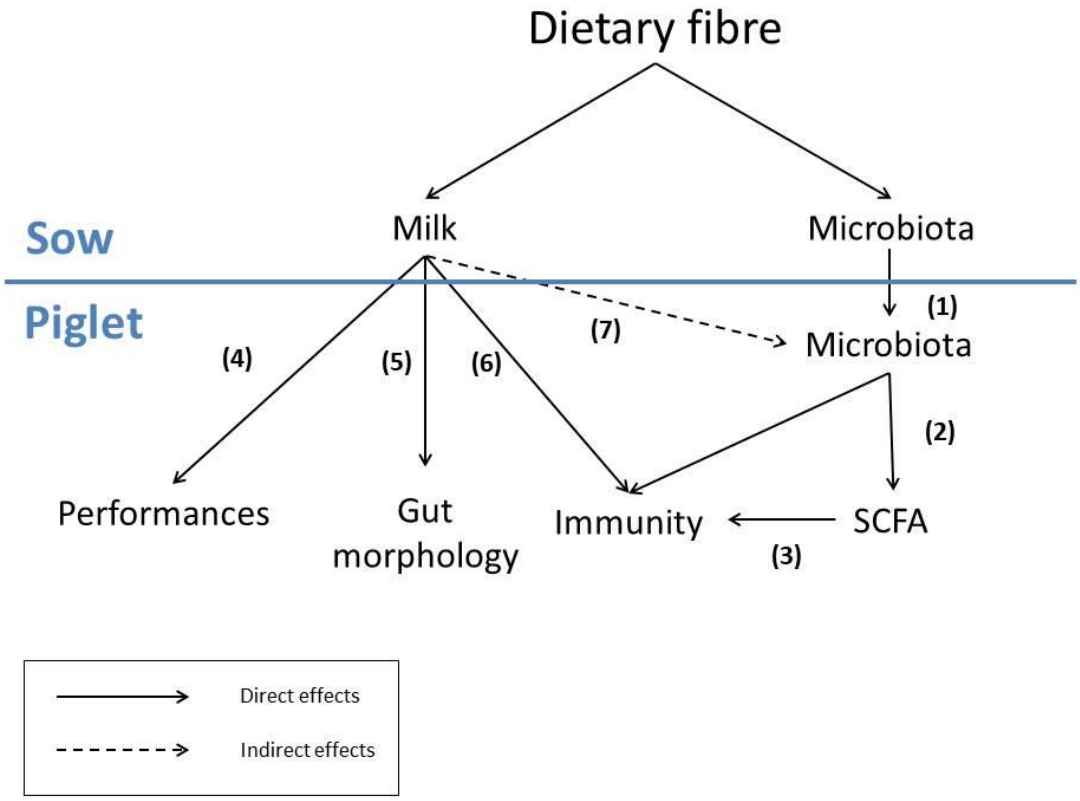


Figure 5. Hypothesis underlying the research question of the thesis.

2. Methods, animal experiments and publications

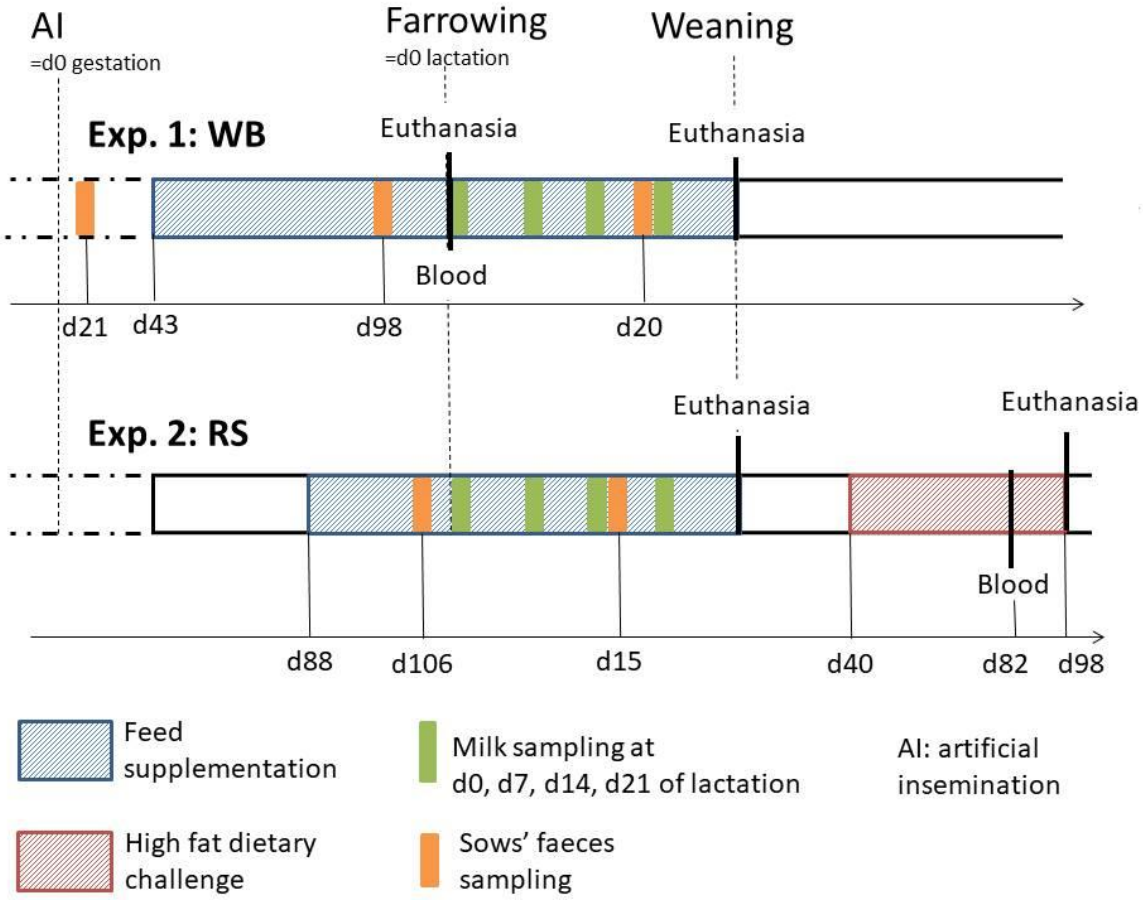


Figure 6. Time line and different sampling points of the two animal experiments performed.

Two animal experiments were conducted, using wheat bran and resistant starch, respectively. The outline for each experiment is represented in Figure 6. For each experiment, faeces of sows were collected before and after parturition in order to determine their faecal microbiota related to the feed used. Milk was collected weekly from birth until the third week of lactation; immunoglobulins concentration and nutrients composition were determined. Prior to weaning, piglets were euthanized and their intestinal contents and tissues collected. The first experiment ended at weaning while the second animal experiment continued until 11 weeks post-weaning, with a 7-weeks high fat challenge and 4 groups of pigs (each maternal diet divided in control or high fat treatments).

Four publications were prepared during this thesis using the data collected during the experiments and are included as chapters of the thesis.

Chapter 3: "Modulation of piglets' microbiota: differential effects by a high wheat bran maternal diet during gestation and lactation" concerns microbiota of sows, piglets and umbilical cord blood and SCFA production of piglets from Exp. 1 (published in Scientific Reports, August 2017).

Chapter 4: "Effects of a high wheat bran diet administrated to sows on performances and intestinal health parameters of the progeny" concerns milk composition, performances and gut morphology and cytokines production of piglets' mucosa from Exp. 1 (submitted to Livestock Science).

Chapter 5: this chapter is not included as a publication but is related to an abstract submitted to EAAP 2017 and presented in the form of a poster: "*In vitro* characterization of different resistant starch sources"

Chapter 6: "Feeding sows pea starch during gestation and lactation impacts their microbiota, milk composition but has little effects of the progeny" includes microbiota and SCFA data of the sows and piglets, milk data, performances, intestinal health at weaning and diarrhoea score at weaning (published in PLoS ONE, July 2018)

Chapter 7: This paper will concern the long-term experiment, including the dietary high fat challenge and the use of the piglets as a model for human obesity. This paper will be submitted when RNAseq results will be available.

Chapter 8 is the general discussion of the thesis.

3

Modulation of piglets' microbiota: differential effects by a high wheat bran maternal diet during gestation and lactation

Article 1 (published in Scientific Reports)

Modulation of piglets' microbiota: differential effects by a high wheat bran maternal diet during gestation and lactation

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Reaching a beneficial intestinal microbiota early in life is desirable for piglets, as microbiota will impact their future health. One strategy to achieve this is the addition of prebiotics to sows' diet, as their microbiota will be transferred. Transmission of microbiota to the offspring occurs at birth and during lactation but a transfer might also occur during gestation. The objectives of this study were to determine whether and when (before and/or after birth) a maternal transfer of the microbiota occurs, and to observe the impact of wheat bran (WB) in sows' diet on their faecal microbiota, their offspring's microbiota and fermentation profile. Sequencing was performed on DNA extracted from umbilical cord blood, meconium, sows' faeces and piglets' colon content. Short-chain fatty acid production was determined in piglets' distal gut. Different bacteria (mostly *Proteobacteria,* followed by *Firmicutes*) were found in the umbilical cord blood, suggesting a maternal transfer occurring already during gestation. Less butyrate was produced in the caecum of WB piglets and a lower concentration of valerate was observed in all intestinal parts of WB piglets. Maternal wheat bran supplementation affected microbiota of sows and piglets differently.

1. Introduction

Intestinal microbiota is acquired early in life and plays multiple roles on host's health : fermentation of fibrous dietary compounds, synthesis of vitamins, maturation of the gut associated lymphoid and immune tissues and resistance to pathogen colonization (Fava et al. 2007; Isaacson & Kim 2012; Kamada et al. 2013; Lawley & Walker 2013; Corsi & Raffatellu 2015). The fermentation of undigested carbohydrates by fibrolytic bacteria within the large intestine leads to the production of short-chain fatty acids (SCFA), mainly acetate, propionate and butyrate (Castillo et al. 2007; Besten et al. 2013) that are used as energy sources by the host. In particular, butyrate is the main energy source for colonocytes and is considered health-promoting due to its anti-inflammatory properties (Guilloteau et al. 2010b). An increase in gut butyrate production might improve host's health and can be beneficial in pig production as piglets are prone to infections especially around the weaning period. In an attempt to apply such a strategy, Ivarsson et al. (2014) observed an increased ileal and faecal butyrate production when feeding growing pigs a high wheat bran (WB) diet (14%) in comparison with other fibre sources (pectin or arabinoxylan sources).

A second factor that might improve piglets' health, possibly on the long term, is the establishment of a beneficial microbiota early in life. This might be done by modulating the sow's microbiota that will be transferred to the offspring. This vertical transfer of the microbiota has been shown both in humans (Thum et al. 2012) and in pigs (Starke et al. 2013; Paßlack et al. 2015). It takes place mainly at birth via a transfer of vaginal and faecal microbes from the mother. However, an earlier mechanism has been more recently unravelled. In humans, a transfer of bacteria has been proven to occur during gestation as bacteria were found in the umbilical cord blood, the meconium, the amniotic fluid (Jiménez et al. 2005; Jiménez et al. 2008) even if the digestive tract of new-borns had always previously been considered as sterile and firstly colonized at birth (Guilloteau et al. 2010a; Besten et al. 2013).

In order to take advantage of this interplay between sows and offspring for pig production purposes, some studies showed that prebiotics in the sow's diet can improve piglets' health status (i.e. greater levels of IgG, IFNγ and activated T cells) by using prebiotics (Leonard et al. 2012; Le Bourgot et al. 2014) but few focussed on the direct impact of sows' diet on the offspring's microbiota (Starke et al. 2013; Paßlack et al. 2015).

Wheat bran (WB) is a source of insoluble non-starch polysaccharides, rich in arabinoxylans, cellulose and lignin that is commonly used in sows' diets for its bulking properties and may be considered as a prebiotic due to its ability to be fermented in the large intestine (Govers et al. 1999; Kamal-Eldin et al. 2009; Bach Knudsen & Canibe 2000). As it has been shown that WB can induce microbiota and

SCFA changes in growing pigs' ileum and faeces (Ivarsson et al. 2014), WB in the maternal diet was used in this study to investigate whether an altered microbiota was observed in sows' faeces at different time points and if this treatment could in turn affect the microbiota and SCFA production of their offspring. Moreover, to investigate *in utero* microbiota transfer, the umbilical cord blood and meconium were collected at birth and analysed for subsequent microbiota determination.

2. Methods

2.1. Animals

The animal experiment and all interventions on animals were approved by the ethical committee of the University of Liège (Belgium, licence number 1661 approved 31st January 2015) and were in compliance with European (directive 2010/63/EU) and Belgian (C − 2013/24221, AR of 23rd of March 2013) regulations concerning the use and care of animals for scientific purposes. The experiment was run at the Walloon Agricultural Research Centre in Gembloux (Belgium). Fifteen Landrace sows, inseminated with Piétrain semen, parity 1 to 5, were divided in two groups, equilibrated for parity, body weight and genetic background.

2.2. Housing

Sows were housed in groups during the gestation period from 3 days after artificial insemination (AI) until 7 days before farrowing. Gestation pens used straw as bedding; the individual farrowing units used wood shavings as bedding.

2.3. Diets and feeding

From day 3 after AI to day 43, all sows received the same gestation diet containing 7% of WB. At day 43, the sows were split in 2 groups and each group was assigned to a dietary treatment, either a control diet (CON, N=7) or a wheat bran-based diet (WB, N=8) until the end of the lactation period (28 days after farrowing). Day 43 was chosen to allow a long adaptation period to the sows and because ultrasound had been performed to confirm gestation of all sows. The same ingredients were used for both the CON and WB diet. WB diet contained 250g/kg DM of wheat bran during gestation and 140g/kg DM during lactation. For a similar feeding phase, diets of both groups were formulated to supply similar amounts of net energy and protein. The composition and nutritive values are given in Supplementary Table ST1 online. Sows were restrictively fed during the gestation period and fed *ad libitum* during the whole lactation period, diets being adapted to their nutritional requirements at each feeding phase (gestation and lactation). Piglets had access to creep feed during the lactation period. The creep feed was devoid of wheat bran, non-starch polysaccharidases and organic acids (composition displayed in Supplementary Table ST2).

2.4. Sample collection

Faeces were collected directly from the rectum of the sows during gestation, 21 days after AI (G21) and 98 days after AI corresponding to 16 days before farrowing (G98+), respectively before and after the dietary change that took place on day 43. Sows' faeces were also collected during the lactation period, i.e. 20 days post-farrowing (L). Faecal samples were placed immediately in sterile bags, snap-frozen and stored at -80°C until DNA extraction. Farrowing was induced by the injection of 2ml of sodium cloprostenol (92µg/ml) at 114 days of gestation. For one piglet during each farrowing, a 5ml sample of umbilical cord blood was collected with a sterile syringe and tube by clamping the cord while the piglet was being born. The same piglet was directly removed from the sows' vulva and euthanized in order to collect meconium in the colon that was snap-frozen and stored at -80°C until DNA extraction. Fourteen blood and meconium samples were collected in total (6 from CON sows, 8 from WB sows). On days 26 and 27 of lactation, 8 female piglets per group (16 in total, 2 piglets/sow, 4 sows/treatment) were euthanized. A mix of Xylazine/Zoletil 100 (4 mg of xylazine, 2 mg of zolazepam and 2 mg of tilamine/kg) was used for anaesthesia followed by T-61 injection directly in the heart (0.1ml/kg) for euthanasia. Their ileal, caecal and colonic contents were immediately collected in sterile tubes, snap-frozen and stored at -80°C until further analysis.

2.5. DNA extraction and sequencing

DNA from sows' faeces, piglets' meconium and colon content was extracted with the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions modified by the addition of two bead-beating steps (FastPrep-24, MP Biomedicals, Illkirsh, France), as described by Yu & Morrison (2004). DNA from umbilical cord blood was extracted with QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The concentration and quality of the DNA were confirmed on a Nanodrop (Thermo Scientific NanoDrop 2000, USA) and by an agarose gel (1%). DNA was then stored at -20°C until sequencing. Sequencing was performed by DNAVision (Gosselies, Belgium), using the Illumina MiSeq (2x300nt) and after amplifying, purifying and tagging the hypervariable regions V3-V4 (Forward primer: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and reverse primer: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') following the 16S Metagenomic Sequencing Library Preparation protocol (Part # 15044223 Rev. B) from Illumina. For sows' faecal DNA, 6 DNA samples per treatment were analysed by sequencing to exclude samples of sows with high parity. For piglets, 7 samples per maternal treatment were analysed based upon the need of high quality DNA for sequencing.

2.6. Short-chain fatty acids (SCFA) and branched-chain fatty acids (BCFA) determination

Piglet's intestinal content and sow's faeces were diluted in ultrapure water to obtain a 6-fold dilution prior to determination of SCFA and lactate by high performance liquid chromatography (HPLC). Piglets' short chain and branched chain fatty acids were analysed by isocratic HPLC, using a Waters system fitted with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) combined with a UV detector (210nm) with sulfuric acid (5mM) as mobile phase at a flow rate of 0.6ml/min. Each peak was integrated by the Empower 3 software (Waters, Milford, USA) after the encoding of the standard curve and then verified manually. The results were expressed in $mg.ml^{-1}$ and were transformed in $mg.g^{-1}$ and $mmol.g^{-1}$, taking into account the initial dilution. The percentages of SCFAs (acetate, propionate, butyrate and valerate), BCFAs (isobutyrate, isovalerate), and lactate were calculated based on the molar ratios. The variable number of samples observed for different intestinal parts is explained by the lack of intestinal contents of some pigs at slaughtering.

2.7. Bioinformatics and statistical analyses

Raw sequences of 16S rRNA were assigned to each sample, quality checked and trimmed using Basespace default parameters (Illumina). Sequences were assigned to 97% ID OTUs by comparison to the Greengenes reference database 13.8 using the QIIME (Quantitative Insights Into Microbial Ecology) 1.9.0 software. Since samples contained variable number of sequences (mean±SEM of 35893±5552 for sows' faeces, 23440±3747 for piglets' colon contents and 209±90 for the umbilical cord blood), diversity analyses were carried out on samples rarefied at the same sequencing depth to avoid bias in sequencing depth between samples. The low number of sequences for the umbilical blood was probably due to the low numerical count of bacteria present in blood in opposition with intestinal contents, as already observed by Vientos-Plotts et al. (2017). The Beta_diversity_through_plots.py script was used to assess differences in bacterial communities and functional composition between groups of samples. Beta diversity was visualized using un-weighed, weighed UniFrac and Bray–Curtis distances with Principal Coordinate Analysis (PCoA). The compare_categories.py script, which applied the adonis method on the previously obtained dissimilarity matrices, was used to determine whether communities differed significantly between groups of samples. Multiple_rarefactions.py and alpha_diversity.py scripts were applied to compute alpha diversity metrics, which evaluated diversity within a sample and generated rarefaction curves. All statistical analyses were performed with SAS 9.2 software (Cary, NC USA). Microbiota results were analysed with the Kruskall-Wallis test which is a non-parametric analysis of variance including multiple comparisons. P-values and false discovery rate (FDR) corrections were determined by the MULTTEST procedure of SAS that calculates the adjusted p-value by using the linear method of Benjamini and Hochberg. SCFA, BCFA and lactate results were analysed with the proc MIXED of SAS, using the

treatment alone (piglets) or treatment and period (sows) as fixed factors. Normality of data (Shapiro-Wilk's test) and variance equality (Levene's test) were checked in SAS prior to analysis. Pearson's correlation coefficient between SCFA ratios and microbiota in colon content were calculated using the PROC CORR of SAS. All data were presented as mean and the SCFA data was presented as mean ± SEM; for all analyses, differences were considered as significant when p-value<0.05 and as substantial when p-values < 0.1.

3. Results

Results of the individual composition of sows' (a) and piglets' (b) microbiota are presented in Figure 7, with a grouping of butyrate-producing bacteria. On the X-axis, each bar chart represents individual animals (ID number on the axis). Because of the important role of butyrate in health-promoting mechanisms of intestinal microbiota, specific attention was paid to the butyrate-producing bacteria in the microbiota analyses. These butyrate-producing bacteria group includes the *Clostridium, Anaerostipes, Blautia, Butyrivibrio, Coprococcus, Dorea, Lachnospira, Pseudobutyrivibrio, Roseburia, Faecalibacterium, Oscillospira, Ruminococcus, Megasphaera* and *Butyricimonas* genera. This group is not exhaustive is based on the classification provided by several articles found in literature (Bian et al. 2016; Louis & Flint 2009; Levine et al. 2013). What is clear from this figure is that there exists a large variability between individual sows even within the same group. To illustrate this, *Prevotella* (in red) can vary for one group (G98+ CON e.g.) from 9% to 25% of the total microbiota. For piglets, the same tendency is observed (from 3% to 16% of the total microbiota is *Prevotella* in the CON group). The same observations can be made for several groups (*Lactobacillus, Bacteroides*).

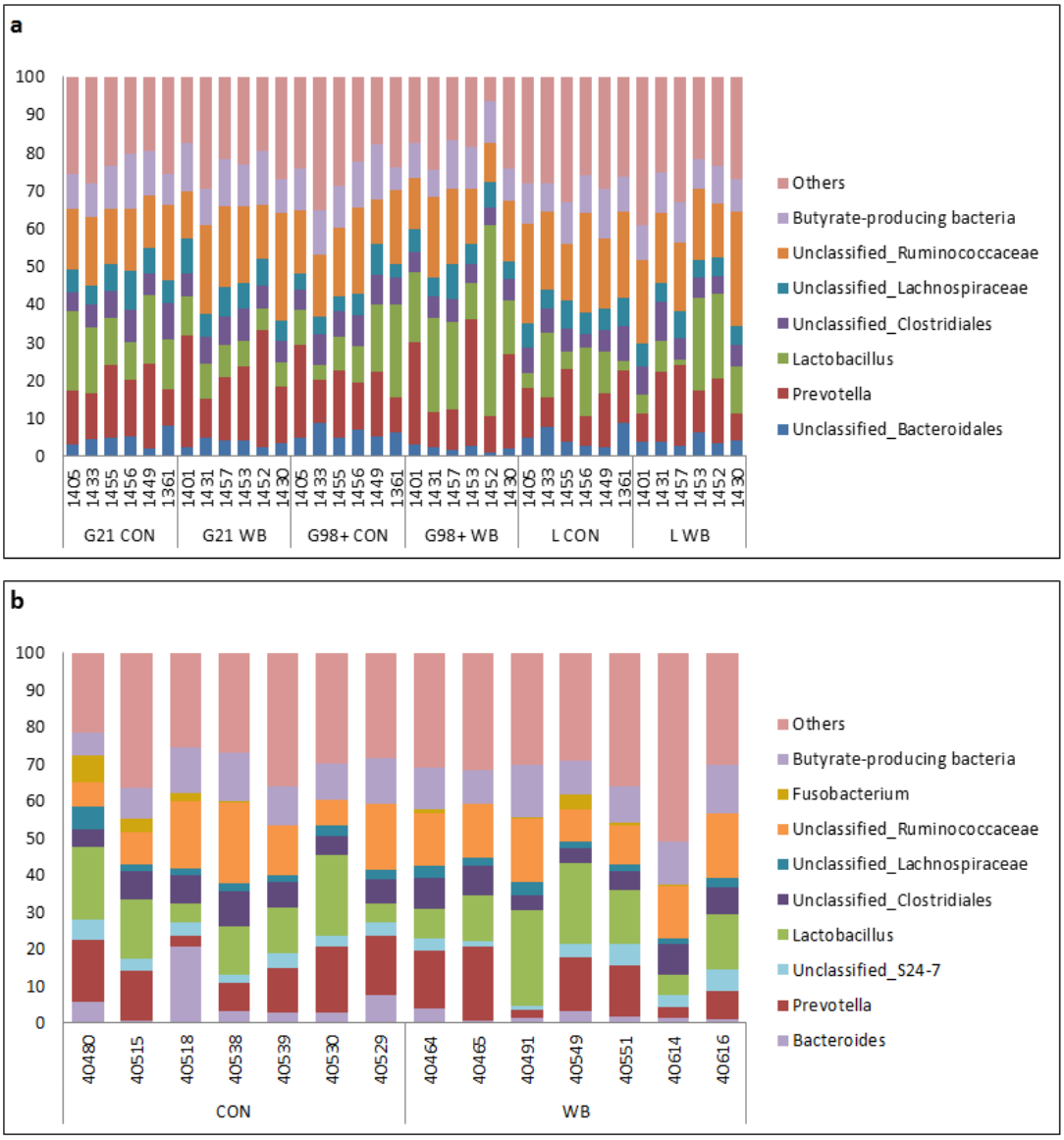


Figure 7. Individual composition of sows' (a) and piglets' (b) microbiota at the genus level. The Y-axis represents the relative abundances of the different genera (expressed as % of the total microbiota) and the X-axis represents the individuals (ID number).

3.1. Sows

The box plot (Figure 8) shows a numerical difference in the calculated Shannon index between CON (7.10) and WB (6.48) samples during G98+, although the Shannon index was not significantly different (p-value=0.06). During G21 and L, the p-value between groups were not significant (p-value=1). The PCoA for sows sampled during gestation when fed the experimental diets shows a clear separation between the CON and the WB groups, the two axes explaining 68 % of the total variability (Figure 8, PCoA based on the weighted Unifrac distance). Such clustering could not be found during the lactation period (see supplementary figure SF1 online).

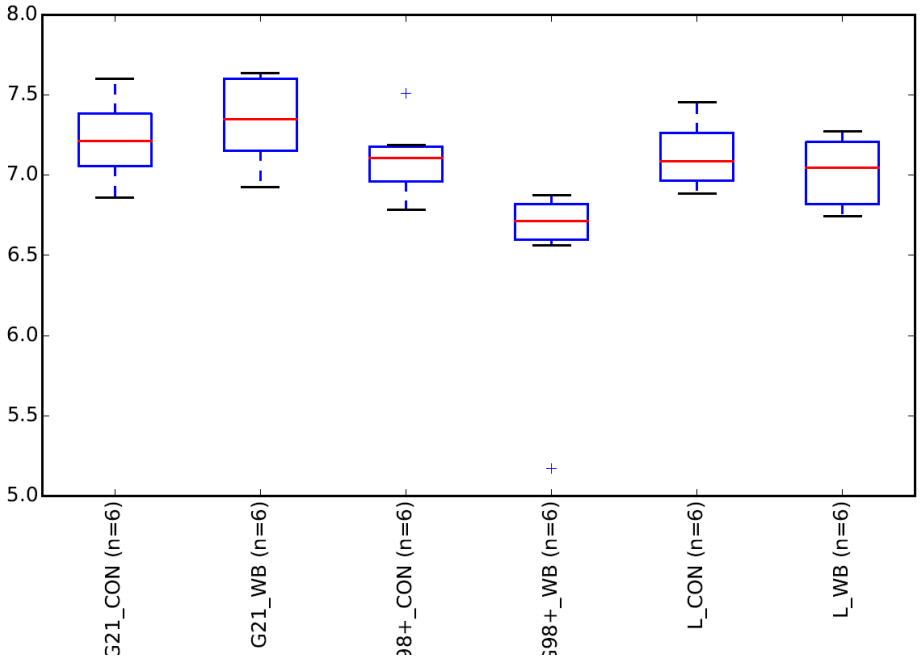


Figure 8. Distribution of alpha diversity as measured by Shannon index, box plots represent the calculated Shannon index for microbiota samples of sows fed the control diet (CON, N=6) and the wheat bran-enriched diet (WB, N=6) at three different stages: 21 d (G21) and 98 d (G98+) of gestation, respectively before and after the experimental diets were distributed, and 20 d of lactation (L).

During the gestation period (G98+), 13 genera differed in relative abundance ($P<0.05$) between the CON and the WB group (Table 3), probably driving the PCoA separation. Most of the genera for which the raw p-value was significant showed a higher abundance in the CON than in the WB group (including Parabacteroides, Unclassified_*Bacteroidales*, Unclassified_RF16, Unclassified_*Clostridiales* and

Oscillospira, a butyrate-producing bacterium), while only Unclassified_*Erysipelotrichaceae* OTU1 were more abundant in the WB group. Only 3 genera showed a significant difference with FDR correction (FDR<0.05, *Parabacteroides*, Unclassified_*Bacteroidales*, SMB53). During lactation (L), two genera, the unclassified RF32 and *Ruminobacter*, showed significant differences ($p<0.05$) between groups, these two being higher in the WB group. It is worth noting that some bacteria, even though non-significantly different between both groups show a numerical difference with a p-value<0.10. Indeed, the butyrate-producing bacterium *Butyrivibrio* is higher in the WB group in lactation compared to the CON group. Before the separation of the two dietary groups (G21), the microbiota of the sows was also analysed. The results can be found in the supplementary table ST3 online. Minor genera (<1% of the total microbiota) were different before the dietary change but were not different afterwards. For *Lactobacillus*, a significant difference was observed: the future CON group had a higher abundance than the future WB group, which is the opposite of what we can observe numerically during G98+.

The SCFA molar ratios of the sows' faeces (see Supplementary Table ST4) were not affected by the dietary treatment whatever the period, while a period-effect was observed ($p<0.001$) for the total SCFA concentration. This value was higher during lactation than gestation.

Table 3. Composition of the faecal microbiota of sows fed the control diet (CON, N=6) and the wheat bran-enriched diet (WB, N=6) at two different stages: 98 d into gestation (G98+) and 20 d into lactation (L), expressed as a percentage (%) of the total mi

Phylum/Genus	G98+				L			
	CON (N=6)	WB (N=6)	P	FDR	CON (N=6)	WB (N=6)	P	FDR
Bacteroidetes	29.3	25.4	NS	NS	23.2	22.7	NS	NS
Parabacteroides	0.36	0.14	**<0.001**	**0.02**	0.36	0.32	NS	NS
Unclassified_*Bacteroidales*	6.13	2.25	**<0.001**	**0.02**	5.1	4.11	NS	NS
Bacteroides	0.22	0.04	**<0.005**	NS	0.12	0.11	NS	NS
CF231	1.22	0.57	**0.01**	NS	0.74	0.69	NS	NS
Unclassified_RF16	2.38	0.79	**0.03**	NS	0.78	0.43	NS	NS
Prevotella	15.5	19	NS	NS	12.7	13.7	NS	NS
Cyanobacteria	0	0	NS	NS	0.07	0.14	0.06	NS
Unclassified_YS2	0.11	0.14	NS	NS	0.07	0.14	0.06	NS
Firmicutes	63.9	67.8	NS	NS	71.2	68.5	NS	NS
Butyrivibrio	0	0.04	NS	NS	0.04	0.14	0.09	NS
SMB53	0.29	0.13	**<0.001**	**0.03**	0.83	0.8	NS	NS
Unclassified_*Lachnospiraceae* OTU2	0.09	0.02	**<0.005**	NS	0.04	0.03	NS	NS
Unclassified_*Clostridiales*	7.17	5.41	**<0.005**	NS	6.23	6.46	NS	NS
Unclassified_ *Erysipelotrichaceae* OTU1	0.02	0.06	**0.01**	NS	0.07	0.06	NS	NS
Anaerovibrio	0.2	0.53	**0.03**	NS	0.3	0.44	NS	NS
Turicibacter	0.13	0.07	**0.03**	NS	0.17	0.12	NS	NS
Oscillospira	2.69	1.76	**0.03**	NS	1.88	1.85	NS	NS
Unclassified_*Erysipelotrichaceae* OTU2	0.08	0.03	0.06	NS	0.05	0.05	NS	NS
Unclassified_*Mogibacteriaceae*	0.75	0.44	0.07	NS	0.46	0.61	NS	NS
Lactobacillus	12.2	23.4	NS	NS	9.47	12.3	NS	NS
Streptococcus	4.84	1.45	NS	NS	6.17	3.72	NS	NS
Unclassified_*Lachnospiraceae* OTU1	5.07	6.19	NS	NS	6.26	5.5	NS	NS
Unclassified_*Ruminococcaceae*	17.5	15.8	NS	NS	21.4	20.3	NS	NS
Unclassified_*Christensenellaceae*	0.55	0.52	NS	NS	3.2	3.02	NS	NS

Continutation of **Table 3.**

Phylum/Genus	G98+				L			
	CON (N=6)	WB (N=6)	P	FDR	CON (N=6)	WB (N=6)	P	FDR
Proteobacteria	1.28	1.5	NS	NS	1.57	2.49	NS	NS
Unclassified_*Enterobacteriaceae*	0.04	0.01	**0.01**	NS	0.31	0.43	NS	NS
Ruminobacter	0.02	0.03	NS	NS	0	0.01	**0.04**	NS
Unclassified_RF32	0.04	0.03	NS	NS	0.02	0.04	**0.05**	NS
Spirochaetes	3.16	3.29	NS	NS	2.3	3.8	NS	NS
Unclassified_Sphaerochaeta	0.33	0.12	0.07	NS	0.18	0.17	NS	NS
Treponema	2.83	3.17	NS	NS	2.12	3.63	0.09	NS

3.2. Umbilical cord blood and meconium

After DNA extraction on the meconium of the new-born piglets, bacterial DNA concentrations were below detection limits and thus could not be sequenced. Consequently, no meconium results can then be presented here. For umbilical cord blood, concentrations of >20ng/µl of DNA were reached for every sample and underwent sequencing. Shannon indexes did not differ between treatments (boxplot displayed in Supplementary Figure SF1, p=0.4). Results (Table 4) showed that different bacteria are present in the umbilical cord blood. *Proteobacteria* accounted for 51.6% and 46.6% of the total bacteria present in the CON and the WB groups, respectively. The second most abundant phylum was *Firmicutes* while Actinobacteria and *Bacteroidetes* were also well represented.

Numerical differences between groups ($p<0.1$) were observed for some genera, including unclassified_*Lachnospiraceae*, which is an intestinal bacterium. Although concentration was low, this bacterium was more abundant in the CON group (0.48%) compared to the WB group (0.08%). Some distal intestinal bacteria were also detected, namely *Corynebacterium, Prevotella*, unclassified_*Bacteroidales*, unclassified_ *Ruminococcaceae* and *Lactobacillus* as well as bacteria colonizing the small intestine (*Psychrobacter, Acinetobacter*). FDR correction did not reveal any differences between treatments.

3.3. Piglets

As insufficient amount of DNA was extracted from the meconium, only results concerning the colonic contents of the piglets at days 26/27 of lactation are presented here. The Shannon index of the microbiota from piglets' colon content did not show any difference in diversity between treatments (boxplot displayed in Supplementary Figure SF2, p=0.6). However, some differences in the relative abundance of genera existed and are presented in Table 5. The most abundant genera in the colon did not differ significantly between both groups but numerical differences were observed for less abundant genera. Indeed, *Colinsella spp.*, a butyrate producing genus, was significantly more abundant ($p<0.05$) in the CON group while *Methanobrevibacter*, unclassified_*Clostridiaceae* (FDR<0.05) and unclassified_*Lachnospiraceae* ($p<0.05$) were more abundant in the WB group. Some genera also exhibited numerical differences between treatments ($p<0.10$), i.e. *Butyricimonas*, *Odoribacter* and *Ruminococcus* were more abundant in the CON group, whereas *Phascolarctobacterium* and *Roseburia* were more abundant in the WB group.

Table 4. Microbial composition of the umbilical cord blood of piglets born from sows fed the control diet (CON, N=6) and the wheat bran-enriched diet (WB, N=8), expressed as the percentage (%) of the total microbiota. Only top ten genera and those with a consistent p-value (<0.1) were included in the table.

Genus	CON (N=6)	WB (N=8)	P	FDR
Actinobacteria	12.4	8.7	NS	NS
Corynebacterium	4.44	1.93	NS	NS
Propionibacterium	6.17	4.76	NS	NS
Bacteroidetes	9.4	16.0	NS	NS
Prevotella	2.60	6.50	NS	NS
Unclassified_*Bacteroidales*	1.06	2.09	NS	NS
Firmicutes	23.1	25.4	NS	NS
Unclassified_*Lachnospiraceae* OTU2	0.48	0.08	0.06	NS
Bacillus	0.48	0.04	0.10	NS
Staphylococcus	3.47	1.24	NS	NS
Unclassified_*Ruminococcaceae*	1.93	2.63	NS	NS
Lactobacillus	6.17	5.34	NS	NS
Solibacillus	0.68	1.93	NS	NS
Streptococcus	1.93	1.01	NS	NS
OD1	0.7	0.0	0.08	NS
Unclassified_ZB2	0.68	0.04	0.08	NS
Proteobacteria	51.6	46.6	NS	NS
Unclassified_*Pseudomonadaceae*	2.80	0.97	NS	NS
Sphingomonas	2.89	2.09	NS	NS
Psychrobacter	12.4	19.3	NS	NS
Acinetobacter	22.1	15.9	NS	NS

Table 5. Relative abundance of bacterial genera sampled in the colon of piglets born from sows fed the control diet (CON) and the wheat bran-enriched diet (WB), only genera with abundance >0.01% are displayed in this table. P-values and FDR are considered as significant <0.05 and numerically different with a value <0.10.

Genus	CON (N=7)	WB (N=7)	P	FDR
Actinobacteria	0.71	0.57	NS	NS
Collinsella	0.29	0.08	**0.04**	NS
Bacteroidetes	32.3	28.4	NS	NS
Butyricimonas	0.15	0.02	0.07	NS
Odoribacter	0.25	0.02	0.07	NS
Bacteroides	6.72	2.21	NS	NS
Unclassified_*Bacteroidales*	3.27	5.61	NS	NS
Prevotella	12.3	11.8	NS	NS
Euryarchaeota	0.01	0.02	**0.05**	NS
Methanobrevibacter	0.01	0.02	**0.05**	NS
Firmicutes	56.0	63.2	NS	NS
Unclassified_*Clostridiaceae*	1.57	2.82	**<0.001**	**0.04**
Unclassified_Lachnospiraceae OTU2	1.91	4.14	**0.04**	NS
Ruminococcus	1.74	0.85	0.07	NS
Phascolarctobacterium	2.35	3.68	0.07	NS
Roseburia	0.11	0.57	0.09	NS
Lactobacillus	14.8	13.1	NS	NS
Unclassified_*Clostridiales*	6.57	6.97	NS	NS
Unclassified_*Ruminococcaceae*	11.7	14.3	NS	NS

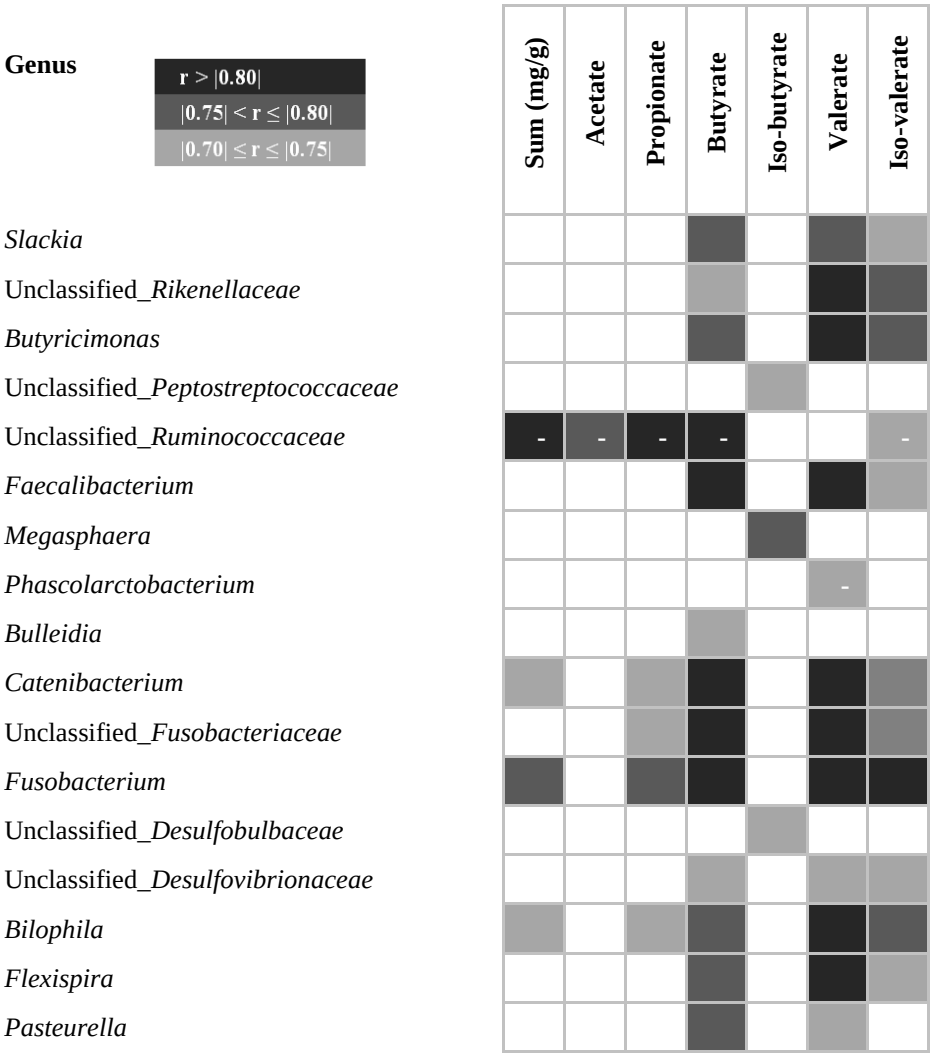
In piglets' intestinal contents, SCFA were affected by the maternal dietary treatment. The molar ratio of acetate was higher in the caecum of WB piglets (57%) compared to CON piglets (51%) and the same tendency (p=0.06) was observed in the colon (Table 6). Butyrate molar ratio was lower in the WB group in the caecum (9.73% in WB vs 13.3% in CON). Valerate molar ratio was lower in the WB group compared to the CON group for each intestinal part. Concerning BCFA, no impact of the maternal dietary treatment was observed, as isobutyrate and isovalerate concentrations were not significantly different for each intestinal part.

Results of Pearson's correlations between SCFA ratios and microbiota data of the colon are shown in Table 7.

Table 6. SCFA concentrations and molar ratios of piglets' digesta in the terminal ileum (n=5 for CON, n=7 for WB), caecum (n=7/treatment) and colon (n=8/treatment). The sum is expressed in mg.g-1 while other compounds are expressed as molar ratios ± SEM.

Intestinal part	Treatment	Total SCFA concentration ($mg.g^{-1}$)	Lactate (%)	Acetate (%)	Propionate (%)	Isobutyrate (%)	Butyrate (%)	Isovalerate (%)	Valerate (%)
Ileum	CON	8.97 ± 5.11	11.1 ± 2.98	30.2 ± 8.21	28.4 ± 11.4	1.81 ± 1.49	13.0 ± 9.36	3.00 ± 1.13	12.5 ± 5.43
	WB	7.51 ± 6.74	18.4 ± 17.4	31.7 ± 16.3	24.4 ± 12.9	1.38 ± 1.32	7.50 ± 5.29	10.4 ± 11.5	6.22 ± 3.38
	p-value	NS	NS	NS	NS	NS	NS	NS	**0.03**
Caecum	CON	10.3 ± 0.40	2.62 ± 0.55	51.1 ± 2.20	20.3 ± 0.73	2.77 ± 0.12	13.3 ± 1.19	3.70 ± 0.50	6.29 ± 0.73
	WB	9.41 ± 0.84	3.45 ± 0.57	57.5 ± 1.27	19.4 ± 0.52	2.70 ± 0.17	9.73 ± 0.85	3.05 ± 0.19	4.22 ± 0.58
	p-value	NS	NS	**0.03**	NS	NS	**0.03**	NS	**0.04**
Colon	CON	7.31 ± 1.26	9.70 ± 2.77	41.5 ± 4.15	19.6 ± 1.28	5.05 ± 2.57	13.3 ± 1.62	3.48 ± 0.33	7.42 ± 0.73
	WB	7.08 ± 0.67	7.28 ± 1.14	51.2 ± 2.28	19.3 ± 0.55	2.38 ± 0.15	11.9 ± 0.92	3.32 ± 0.27	4.63 ± 0.55
	p-value	NS	NS	0.06	NS	NS	NS	NS	**0.01**

Table 7. Pearson's correlations between SCFA molar ratios and genera of the microbial community in the colon of piglets from sows fed a control and a wheat bran-enriched diet (N=14). Only the results with a p-value<0.05 and r>0.70 were included in this table. Negative correlations are expressed in the table with the symbol "-".



4. Discussion

The aim of the study was to investigate whether the addition of wheat bran in the diet fed to gestating and lactating sows would alter their intestinal microbiota and if this diet would in turn alter their offspring's microbiota and subsequent SCFA production. Moreover, the study aimed at determining whether a maternal transfer of microbiota occurred already *in utero*. Regarding the latter and to the author's best knowledge, it is the first time in pigs that a maternal transfer occurring during the gestation period is reported, as shown by the umbilical cord blood microbial results. Similar results have been observed in humans as Jiménez et al. (2005) isolated four bacterial genera from the umbilical cord blood: *Enterococcus, Propionibacterium, Staphylococcus,* and *Streptococcus*. The three last genera had also a high share in the microbial communities of umbilical blood in this experiment with pigs, strengthening the reliability of our results. In this experiment, more than four genera have been observed, which is probably due to the direct DNA extraction from blood instead of the pre-culturing of blood that was done by Jiménez et al. (2005). As no bead beating step was added for the umbilical blood, it may be possible that more bacterial DNA could be extracted by adding bead beating steps during the DNA extraction. The presence of intestinal bacteria in the umbilical cord blood suggests a microbial transfer from the mother to the offspring already during gestation. So it can be surprising that no detectable DNA was found in the meconium. A first explanation for this lack of detectable DNA in the meconium is that some bacteria found in the umbilical cord blood are not hosted in the colon lumen (i.e. meconium collected) but in the small intestine like *Psychrobacter* and *Acinetobacter* (Zhao et al. 2015) or in the intestinal mucus layer, like *Proteobacteria* and *Ruminococcaceaeae* (Tran et al. 2015) and could thus have colonized these locations that were not sampled in this study. Also, as the kits used for blood and meconium DNA extractions were not the same, it might be that the kit used for meconium may not be sensitive enough to quantify small amounts of DNA. Other evidences of maternal bacterial transfer during gestation could be found by the analysis of mucus layer and small intestine of the piglets and the use of a more sensitive kit to extract DNA from the meconium. To understand better the mechanism of the maternal transfer during the gestation period, sampling of amniotic fluid that is directly ingested by the foetus in the second part of the gestation (Guilloteau et al. 2010a) could be interesting in the future as well as placenta during C-section.

Regarding the main hypothesis in this experiment, namely the introduction of WB into sows' diet and the impact on their microbiota, it was shown that during gestation, 13 genera abundances differed in sows' faeces between both dietary groups and 2 additional genera differed during lactation. Only during gestation (G98+), a nice clustering of the two groups was observed with the PCoA, most probably due to the genera for which p-values were different.

Ivarsson et al. (2014) found that lactic acid bacteria in faeces of growing pigs fed a 14% WB diet were significantly higher while *Prevotella* were lower. This tendency was not observed in the current study for *Prevotella* while *Lactobacillus* relative abundances were higher during G98+ and L in the WB group without being statistically significant, probably due to a high variability between individual sows, which is in agreement with studies highlighting the individual variations (Kim et al. 2011). Seen the variability between individuals, it can be interesting for future research to increase the number of animals with the same genetic background and equal parity. In this study, results showed a higher proportion of *Oscillospira*, which has been reported to be a butyrate-producing genus (Upadhyaya et al. 2016), in the CON group during gestation. However, a negative correlation (see Supplementary table ST5 online, r= -0.57) has been observed between butyrate production and the abundance of *Oscillospira* for piglets, suggesting that this genus can have a different impact on butyrate production depending on the species or strains. Most of the differences in sows' faecal microbiota occurred for minor groups of microbiota present in the faeces of sows (<1% of the total microbiota). However, some genera with a different abundance amongst treatments were well-represented in the microbial community, i.e. *Oscillospira* (2.7% in the CON group, 1.8% in the WB group), the unclassified *Bacteroidales* (6.1% in CON, 2.2% in WB) and *Clostridiales* (7.2% CON, 5.4% WB). During the lactation period, the butyrate-producing genus *Butyrivibrio* was increased in the WB group (0.14%) compared to the CON group (0.04%). Different studies (Chen et al. 2013; Chen et al. 2014; Yu et al. 2016) showed an increase in *Bifidobacterium* in the distal part of the gastrointestinal tract of growing pigs fed by a WB diet, but this was not observed for sows in the present study. It must be emphasized that most studies on WB have been performed on piglets or growing pigs, which renders the comparison difficult as the microbiota is related to age (Bian et al. 2016) and encounters changes as the pig grows (Kim et al. 2011).

The differences in the microbial composition of sows' faeces observed for the two dietary treatments in gestation were less pronounced in lactation, where only 2 genera were different between both groups, illustrated by the lack of clustering between groups for PCoA in opposition with gestation. The first possible explanation for this resides in the lower amount of WB in the diet that could be included to meet the nutritional requirements of the sows during the lactation period. A second hypothesis is that the gestation and lactation diets contained different ingredients in different proportions, such as soya pods that can be fermented. Thirdly, microbiota composition is not stable and can vary with physiological stages. It is particularly true for piglets for which colonization by microbiota has been demonstrated to be affected by stress (Schokker et al. 2014) but may apply to sows as farrowing and lactation are stressful periods due to handlings on sows and piglets and accompanied with physiological changes. A last explanation could be the different environment in gestation and lactation, as the bedding materials differed.

The maternal dietary treatment impacted the composition of the microbiota in piglet's colon, which was distinct from the sow's faecal microbial alterations. This was also observed by different studies when feeding sows with inulin or probiotics (Starke et al. 2013; Paßlack et al. 2015). As highlighted by Paßlack et al. (2015), the discrepancy between microbiota changes in sows and piglets can be ascribed to the use of faeces for sows and colon content for piglets, which may not be the exact reflection of a sow's colon. Another explanation for these differences resides in the fact that as diet is a major driver for microbiota composition, the microbiota is probably the reflection of their different diets (solid diet for sows *vs* milk for piglets). Moreover, the piglet acquires not only the faecal microbiota from the sow but also microbial communities present in the vagina, on the skin and in the environment of its mother. All these observations probably contributed to some extend the discrepancies between sow's and piglet's microbiota. Furthermore, besides a faecal transfer, the colostrum and the chemical and microbial composition of milk might as well influence the intestinal microbiota of the progeny (Bian et al. 2016) which is worth to be investigated. As microbiota composition is related to age, it would be interesting to analyse pigs' microbiota at the adult age to see if and how these discrepancies evolve, as a stable microbiota was not reached at our sampling time period (26 days of age, Bian et al. 2016).

SCFA production in piglets' intestinal contents was measured and differences between groups were observed. In the caecum and colon, acetate was higher for WB piglets compared to CON animals, whereas valerate was lower for WB piglets for each intestinal part. The butyrate production was higher in the CON pigs in the caecum, which was unexpected but no support in literature could be found on indirect impact of maternal WB on progeny's butyrate production. Some plausible explanations for these results can be found in the correlation matrix between microbial genera and SCFA production in the colon. Indeed, the lower valerate production in the WB group can be partly explained by the higher abundance of genera with a negative correlation with valerate production: the unclassified_*Bacteroidales* (r=-0.55), the unclassified_*Clostridiaceae* (r=-0.63), unclassified_*Lachnospiraceae* OTU2 (r=-0.58) and *Phascolarctobacterium* (r=-0.73). Moreover, some valerate-producing genera, as determined by the correlation matrix, were more abundant in the CON piglets than in the WB: *Colinsella* (r=0.57) and *Odoribacter* (r=0.69). *Butyricimonas* was positively correlated with butyrate (r=0.80), iso-valerate (r=0.75) and valerate (r=0.86) production, explaining partly the higher valerate and butyrate production of the CON piglets as this genus was significantly more abundant than in the WB piglets. The lower valerate concentration could be considered as beneficial for health, as valerate is an end-product of protein fermentation and can lead to the production of toxic compounds, even though the impact of valerate on the colon is poorly documented (Mortensen et al. 1992; Walton et al. 2012; Yao et al. 2016; Poelaert et al. 2017). The higher acetate production in the WB group does not seem to increase butyrate production (bacteria can use acetate

as alternative pathway to produce butyrate) but can be further absorbed by colonocytes as energy source and taken up by the liver for energy purposes (Besten et al. 2013).

5. Conclusion

In conclusion, this study showed that a maternal transfer is possible, and that it might already take place during gestation, as seen by the microbiota composition of the umbilical cord blood. The maternal diet impacted the piglet's microbiota and fermentation end-products profile, even though, conversely to what was expected, the butyrate did not increase in the WB piglets. In a more holistic approach for future studies, it would be interesting to investigate long-term effects on piglet's microbiota and health.

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Author contributions

Experimental set-up and design: J.L., J.W., J.B., N.E.; Bioinformatic analyses and statistics of sequencing results: S.M.; Practical analyses: J.L.; L.B.; N.E.; Manuscript preparation: J.L., N.E. All authors read and approved this article.

Additional information

Supplementary tables and figures can be found in the online supplementary material.

The authors declare no competing financial interests in this study.

Data availability

Raw sequences can be found on the ENA (European Nucleotide Archive) database, under the project accession number ERP023150.

Appendix 1: Supplementary material article 1

Modulation of piglets' microbiota: differential effects by a high wheat bran maternal diet during gestation and lactation

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Supplementary Table ST1. Composition and nutritive values of sows' diets.

Ingredients (%)	GC	GWB	LC	LWB
Wheat	19.993	15.3	23.954	17.942
Maize	15	12	12	12
Barley	15	10	11.4	10
Wheat bran	-	25	-	14
Soya	-	-	13.7	13.1
Bread flour	6.5	5	6.5	5
Sugar beet pulp	5.5	5.5	5.5	5.5
Biscuit flour	5	3.5	5	4
Cocoa pods	5	1.5	3.9	0.8
Sunflower meal	9	4.2	2.5	1
Palmist meal	4	4	1.2	1.6
Soya pods	1.9	1.9	3	3
Treacle	2	1	3	3
Nutex 68 (Dumoulin Inc)	3.6	3.3	3.3	3.3
Chalk	1.17	1.21	1.61	1.66
Lard	0.47	1.25	1.47	2.25
Rapeseed meal	3	2.2	-	-
Rapeseed flour	1.2	1.2	-	-
Soya oil	-	0.55	0.05	-
Minerals & Vitamins	1.242	0.982	1.41	1.34
L-Lysine 50 %	0.386	0.372	0.393	0.385
L-threonine	0.039	0.038	0.081	0.09
DL-methionine	-	-	0.032	0.033
Thr+Met 70/30	-	0.034	-	-
DM	88.78	88.82	88.3	88.3
Crude ash	5.91	5.71	6.24	6.07
OM	94.09	94.29	93.76	93.93
CP	14.59	14.55	18.09	17.4
ADF (%)	11.89	11.43	9.88	9.75
NDF (%)	22.41	25.41	19.73	20.93
Starch (%)	34.06	30.56	34.21	31.15
Fat (%)	5.53	7.22	6.12	6.97
GE (kcal/kg MS)	4520	4633	4508	4613

WB = wheat bran; GC = gestation, control diet; GWB = gestation, WB diet; LC = lactation, control diet; LWB = lactation, WB diet

DM = analytical dry matter; OM= organic matter; CP = crude protein; GE = gross energy; ADF = acid detergent fiber; NDF = neutral detergent fiber

Supplementary Table ST2. Piglets' creep feed composition.

Ingredient (%)	Creep feed
Maize flaked	44.9
Skimmed milk	23.5
Soybean	10.0
Soybean meal	10.0
Maize starch	5.0
Soybean oil	2.2
Vit:min	1.0
Monocalciumphosphate	0.9
Cellulose	0.8
Inert markers	0.5
L-lysine HCl	0.4
Salt	0.3
Phytase	0.2
L-Threonine	0.1

Supplementary Table 3. Composition of the faecal microbiota of sows before the diet change (G21), expressed as a percentage (%) of the total microbiota. Only genera with a relative abundance >0.01% were included in this table.

Genus	CON	WB	P-value	FDR
Bacteroidetes				
Prevotella	15.3	20.3	NS	NS
Unclassified_*Lachnospiraceae*	6.92	6.97	NS	NS
Firmicutes				
Unclassified_*Ruminococcaceae*	16.4	19.9	NS	NS
Lactobacillus	15.3	7.8	**0.002**	NS
Unclassified_*Clostridiales*	6.91	6.81	NS	NS
Ruminococcus	2.68	2.55	NS	NS
Blautia	0.17	0.32	0.04	NS
Bacteroides	0.11	0.02	0.03	NS
Bulleidia	0.07	0.13	0.03	NS
Proteobacteria				
Treponema	2.92	2.12	0.08	NS
Phascolarctobacterium	1.93	1.88	NS	NS
ParaPrevotella	0.02	0.00	0.01	NS
Spirochaetes				
Unclassified_*Bacteroidales*	4.75	3.66	NS	NS
Oscillospira	2.76	2.30	NS	NS
TM7				
Unclassified_*Enterobacteriaceae*	0.09	0.01	0.07	NS
WPS-2				
Sphaerochaeta	0.32	0.15	0.05	NS

Supplementary Table ST4. Sows' faecal SCFA production. The individual SCFA are expressed as molar ratios. Results are mean ± SEM.

Period	Treatment	N	Sum ($mg.g^{-1}$)	%lactate	%acetate	%propionate	%isobutyrate	%butyrate	%isovalerate	%valerate
G21	CON	7	6.96 ± 0.94	2.56 ± 1.08	58.6 ± 3.54	20.83 ± 1.64	2.41 ± 0.38	10.4 ± 1.27	2.98 ± 1.10	2.24 ± 0.64
	WB	8	7.71 ± 1.75	2.48 ± 1.99	57.9 ± 2.35	20.8 ± 2.45	2.10 ± 0.73	11.9 ± 1.68	2.57 ± 1.27	2.29 ± 0.77
G98+	CON	7	8.71 ± 1.66	2.43 ± 1.45	57.0 ± 2.97	22.3 ± 3.62	1.95 ± 0.61	11.1 ± 1.97	2.58 ± 1.18	2.73 ± 0.76
	WB	8	8.50 ± 1.28	2.86 ± 1.18	56.5 ± 4.83	21.1 ± 1.95	2.13 ± 0.85	11.1 ± 0.71	3.24 ± 1.60	3.11 ± 0.83
L	CON	7	11.3 ± 2.42	1.88 ± 0.95	57.4 ± 1.72	21.4 ± 1.03	2.38 ± 0.51	10.9 ± 1.21	3.50 ± 0.90	2.53 ± 0.58
	WB	8	12.1 ± 1.97	1.84 ± 0.97	55.9 ± 1.37	23.5 ± 1.62	2.24 ± 0.22	10.9 ± 1.34	3.14 ± 0.72	2.45 ± 0.37
P-values	T		0.413	0.721	0.303	0.715	0.604	0.24	0.916	0.45
	P		<.0001	0.199	0.241	0.014	0.498	0.732	0.194	0.182
	T*P		0.389	0.835	0.864	0.05	0.547	0.078	0.446	0.34

G21: gestation before diet change, G98+: lactation after diet change, T= treatment, P= period

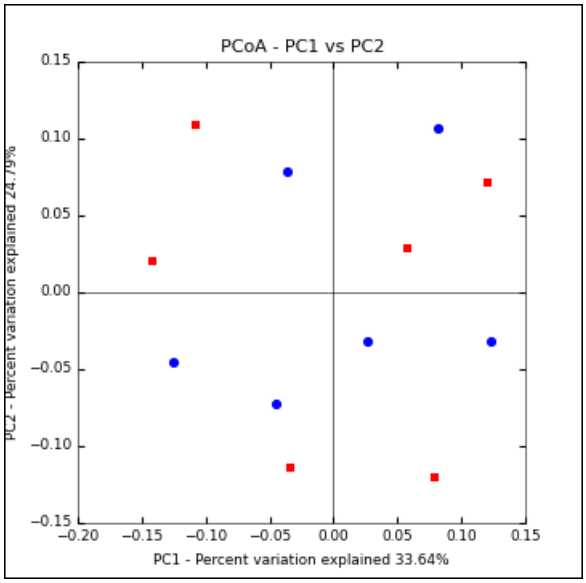
Supplementary Table ST5.Pearson's correlations between SCFA molar ratios and genera of the microbial community in the colon of piglets from sows fed a control and a wheat bran-enriched diet (n=14). Only the results with a p-value<0.05 were included in this table. Negative correlations are expressed in the table with the symbol "-".

Genus	Sum (mg/g)	Acetic Acid	Propionic acid	Butyric acid	Isobutyric acid	Valeric acid	Isovaleric acid
Unclassified_Microbacteriaceae		-					
Leucobacter		-					
Propionibacterium							-
Collinsella							
Slackia							
Unclassified_Rikenellaceae							
Unclassified_[Barnesiellaceae]							
Butyricimonas							
Odoribacter							
Unclassified_[Para*Prevotella*ceae]		-					
Unclassified_Stramenopiles		-					
Leuconostoc	-		-	-		-	-
Unclassified_Clostridia		-					
Unclassified_Clostridiales	-	-	-				
Unclassified_Christensenellaceae	-		-	-		-	
Christensenella							
Unclassified_Clostridiaceae						-	
SMB53	-	-	-	-			
Unclassified_Lachnospiraceae							
Coprococcus					-		
[Ruminococcus]					-	-	
[Clostridium]							
Unclassified_Ruminococcaceae	-	-	-	-		-	-
Anaerotruncus							
Clostridium							
Faecalibacterium							
Oscillospira				-			
Megasphaera							
Phascolarctobacterium						-	

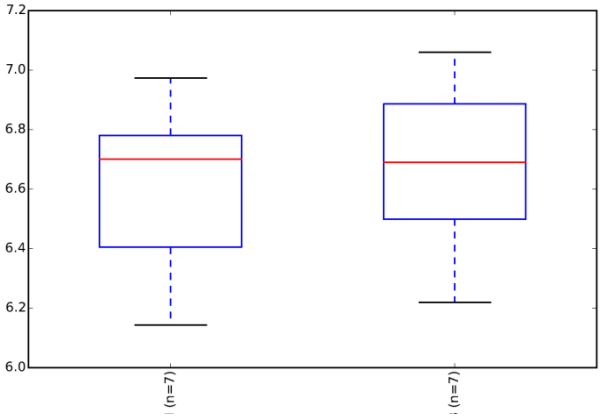
0.51	< r <	0.60
0.61	< r <	0.70
0.71	< r <	0.80
0.81	< r <	0.90

Continuation of **Supplementary Table ST5.**

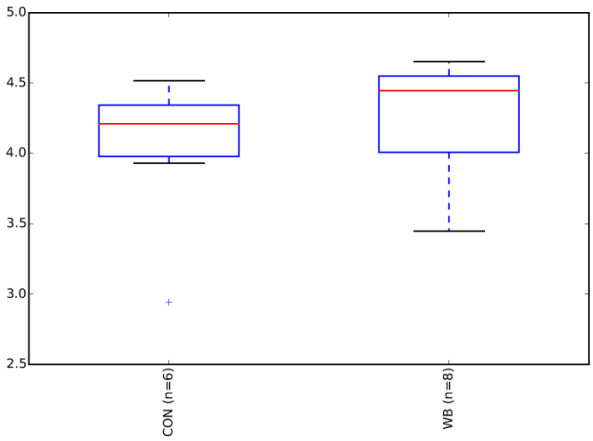
Genus	Sum (mg/g)	Acetic Acid	Propionic acid	Butyric acid	Isobutyric acid	Valeric acid	Isovaleric acid
Veillonella							
Anaerococcus							
Bulleidia							
Catenibacterium							
L7A E11	-	-					
[Eubacterium]							
Unclassified_Fusobacteriaceae							
Fusobacterium							
Sphingomonas							
Sutterella							
Unclassified_Desulfobulbaceae							
Unclassified_Desulfovibrionaceae							
Bilophila							
Flexispira							
Helicobacter							
Klebsiella							
Pasteurella							
Sphaerochaeta						-	-
Treponema			-				
Unclassified_RFP12	-			-		-	



Supplementary Figure SF1. PCoA of microbial communities of sows fed the control diet (CON, N=6) and the wheat bran-enriched diet (WB, N=6) during lactation. Individual WB sows are displayed in red and CON sows in blue.



Supplementary Figure SF2. Boxplot based on Shannon index for the umbilical cord blood microbial results.



Supplementary Figure SF3. Boxplot based on Shannon index for the piglets' microbial results.

4

Effects of a high wheat bran diet administrated to sows on performances and intestinal health parameters of the progeny

Article 2 (Submitted in Livestock Science)

Effects of a high wheat bran diet administrated to sows on performances and intestinal health parameters of the progeny

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Short title

Maternal wheat bran diet on piglets' health

1. Abstract

As weaning is a critical period in pig production, there is a need to find sustainable alternatives to the use of antibiotics to prevent or cure post-weaning diarrhoea. In this study, we hypothesized that the supplementation of sows with high amounts of wheat bran (**WB**) in gestation and lactation can modify milk composition and improve piglets' health. An animal experiment was run with 15 sows, divided in two groups, a WB group (receiving 25% or 14% of wheat bran during gestation and lactation, respectively) or a control (**CON**) group devoid of WB. Backfat thickness and bodyweight changes, litter size, number of weaned piglets and weekly bodyweight of the piglets were recorded to assess performance of the animals. Before weaning, 16 female piglets (8/group) were euthanized; their intestinal tissues and contents were collected. An *ex vivo* lipopolysaccharide (**LPS**) challenge was run on ileal tissue to assess a differential expression of cytokines. No impact of the dietary treatment was observed on sows' backfat or bodyweight changes, but a decrease of the feed intake was observed during the last period of lactation for the WB group. The bodyweight of the piglets from birth until weaning was not affected by the maternal dietary treatment. Colostrum and milk immunoglobulins (IgA, IgG, IgM) were similar between groups during the whole lactation period. In milk, protein and fat contents were not affected by the dietary treatment while maternal WB diet increased the lactose content. Villus height was increased in the duodenum of piglets born from WB sows as well as the ratio villi/crypts in the duodenum and jejunum, suggesting a higher absorption of nutrients for piglets born from WB sows. Calprotectin and intestinal inflammation (TNF-α, TLR4) did not differ between piglets born from the two maternal diets. In conclusion, increasing the WB proportion in sows' diet did not decrease their performances or their piglets' health, while improving lactose concentration in sows colostrum and milk and gut morphology possibly affecting the absorptive capacity of the piglets' gut. Therefore, diets enriched in WB can be used during gestation and lactation.

Keywords

Fibre, intestinal health, maternal transfer, piglets, weaning

2. Introduction

Weaning is a critical moment in pig production due to multiple stressful events occurring simultaneously: separation from the sow, mixing with other litters, abrupt transition from milk to a solid feed diet, change in the environment and greater exposure to pathogens (Campbell et al., 2013). As a result, piglets show low feed intake and experience gut disorders after weaning, such as shortening of intestinal villi and deepening of crypts, a lower brush border enzyme activity, a higher permeability to antigens in the gut mucosa and an altered immune response (Campbell et al., 2013; Lallès et al., 2004; Montagne et al., 2007) . Ultimately, weaning causes loss of weight of the piglets often associated with the appearance of post-weaning diarrhoea (**PWD**) that is mainly due to colibacillosis (Gresse et al., 2017).

Even though the use of antibiotics as growth promoters has been banned by the EU in 2006 (EU IP/05/1687), antibiotics are still used in piglet production both to prevent and treat PWD. Concomitantly, overuse of antibiotics has led to the appearance of multi-resistant bacterial strains threatening animal and public health (Pluske 2013; Lindberg 2014; Gresse et al. 2017). Therefore, more sustainable alternatives to improve piglets' health are desirable and various nutritional approaches have been proposed to help piglets cope with the weaning transition, including the supplementation of the diet with substances that increase appetence, have immune-stimulating properties or target the intestinal microbiota (Lallès et al. 2004; Devriendt et al. 2009). Since the intestinal microbiota is involved in the maturation of the immune system of piglets, one strategy is to act on sows diets to alter the composition of the intestinal microbiota of the sow, subsequently modifying favourably the microbiota colonizing the piglets early in life (Everaert et al., 2017; Schokker et al., 2014).

Wheat bran is widely used in gestation diets of sows. Thanks to its bulking properties, it reduces frustration from restrictive feeding and increases welfare (Matte et al., 1994). However, the incorporation rate of WB in sows' gestation diets stays moderate. Moreover, as WB is an ingredient rich in insoluble non-starch polysaccharides, it is fermented in the colon by the intestinal microbiota (Bach Knudsen and Canibe, 2000; Govers et al., 1999) and has been shown to stimulate beneficial bacteria in the ileum and faeces of growing pigs (Ivarsson et al., 2014) while decreasing the faecal score of piglets after weaning (Francesc Molist et al. 2012). In addition, it has been shown that a high WB diet given to sows impacted their microbiota during gestation and a few genera of their offspring's colonic microbiota at weaning (Leblois et al., 2017). This transfer of microbiota from the sow to the piglets takes place during gestation, at birth and during the lactation period (Leblois et al., 2017; Paßlack et al., 2015; Starke et al., 2013). As gut bacteria will modulate the maturation of the immune system early in piglets' life (Schokker et al.,

2014), it is possible that a change in sows' and piglets' microbiota could impact the inflammatory response of piglets facing a bacterial infection.

In addition, wheat bran, as a potential prebiotic, might impact several milk components and piglets parameters. Indeed, other prebiotics have been shown to affect positively the immunoglobulin concentration of colostrum (Le Bourgot et al., 2014; Leonard et al., 2012), which confers a systemic or local protection against pathogenic antigens. Moreover, the nutritional composition of sows' milk can be altered by the inclusion of prebiotics in sows' diet (Graugnard et al., 2014; Krogh et al., 2017). Concerning piglets' intestinal parameters, Le Bourgot et al. (2014) showed an increased IFNγ concentration and an increased number of T-helper lymphocytes in Peyers' patches of piglets born from fructo-oligosaccharide (**FOS**) supplemented sows, and Heim et al. (2015a) observed a decreased villus height of the ileum of piglets born from fucoidan supplemented sows, which was related to a lower expression of glucose transporter SGLT1. The hypothesis tested in the current study was that incorporation of high amounts of WB in sows' diet would modify their milk composition that in turn affects piglets' performances, immunity and gut morphology. Gut morphology and inflammation being also related to microbiota and subsequent short-chain fatty acids production, the latter two already described in Leblois et al. (2017).

3. Materials and methods

3.1. Animals, diet and housing

The animal experiment was approved by the Ethical Committee of the University of Liège (protocol number 1661) and the procedure agreed with the European (directive 2010/63/EU) and Belgian (C − 2013/24221, AR of 23rd of March 2013) regulations. The animal experiment was led at the Walloon Agricultural Research Centre (Gembloux, Belgium). Fifteen Landrace sows (parity 1 to 5) were allotted to two dietary treatments, the groups being balanced for parity and genetic background. Sows were housed in loose groups on straw bedding (room was separated in two units to avoid faeces contamination) from day 3 after artificial insemination (**AI**) and then moved one week before parturition to individual farrowing cages equipped with wood shavings, a heat lamp for piglets and extra space for sows and piglets available from day 5 after delivery. Water was supplied at libitum with drinker bowls. Parturition was provoked by the injection of 2 ml of sodium cloprostenol (92 µg/ml) at 114 days of gestation.

Two diets, a control diet (**CON**, 7 sows, 3 1st parity sows, 4 multiparous sows) devoid of wheat bran or an enriched-wheat bran diet (**WB**, 25% of WB in gestation and 14% in lactation, 8 sows, 4 primiparous sows, 4 multiparous sows), were fed to the sows from the 43rd day of gestation onwards to allow the longest time for microbiota adaptation and after gestation was confirmed. During gestation, sows

were fed-restricted according to their bodyweight and parity, and during lactation they were fed *ad libitum*. For each feeding phase, diets were formulated to be iso-energetic and iso-nitrogenous and to reach the nutritional requirements of the animals following the NRC recommendations (Table 8). Piglets had access to creep feed that was devoid of wheat bran, non-starch polysaccharidases and organic acids from day 10 after birth. Sows' feeders were adapted to prevent piglets from eating the sow's diet. The lactation period lasted for 28 days.

3.2. Zootechnical performances

Sows were weighted and backfat thickness was recorded with a lean-meater ® (Secrepro, Québec, Canada) 4 days before AI, during gestation (day 38 and day 108) and at weaning. Backfat and bodyweight gain/loss were assessed between these periods. The number of piglets born alive and the number of piglets weaned were recorded. During the lactation period, each piglet was weighted weekly, from birth until weaning. Feed intake of the sows was measured daily during the lactation period, with a computerized feeding system (Gestal FM ®, Jyga Technologies, Canada). Each sow had a target ingestion curve (target DM intake/day during the whole lactation period), as they had a maximal feed intake depending on their parity. Data shown are the percentage of this curve reached, in order to take into account the different parities. The first days of lactation are not taken into account as sows' ingestion is close to 0.

3.3. Milk

Colostrum (15 ml) was sampled manually at all functional tits within the first 3 hours after the beginning of the farrowing (birth of the first piglet). Milk (15ml) was then sampled weekly after the intramuscular injection of 1.5ml of oxytocin 10Un./ml (V.M.D., Belgium). Colostrum and milk were filtered on sterile medical gauze and stored at -20°C until further analyses. Samples were analysed for fat, protein and lactose content by Fourier transform infrared spectroscopy on a Standard Lactoscope FT-MIR automatic (Delta Instruments, Drachten, The Netherlands) as already done by Decaluwé et al. (2013). The predictive models provided by the manufacturer were originally designed for analysis of cow milk and were consequently updated for sow milk by a slope and bias correction using a set of sow milks with known reference values obtained by chemical reference analysis. Immunoglobulins (IgA, IgG, IgM) concentrations were determined by specific anti-pig antibodies *ELISA* (Bethyl Laboratories, Montgomery, USA and R&D Systems, Oxon, UK), following the manufacturer's recommendations. 3,3',5,5'-Tetramethylbenzidine was used as developer of the reaction (Fisher Scientific, Merelbeke, Belgium) and the reading of the plate was made at 450µm on a 96-wells plates reader (Stat-fax 2100, awareness technology Inc, USA).

Table 8. Ingredients proportions and analysed chemical composition of gestation and lactation diets of the sows.

Ingredients (%)	**GC[1]**	**GWB**	**LC**	**LWB**
Wheat	20.0	15.3	23.9	17.9
Maize	15.0	12.0	12.0	12.0
Barley	15.0	10.0	11.4	10.0
Wheat bran	-	25.0	-	14.0
Soybean grains	-	-	13.7	13.1
Bread flour	6.50	5.00	6.50	5.00
Sugar beet pulp	5.50	5.50	5.50	5.50
Biscuit flour	5.00	3.50	5.00	4.00
Cocoa pods	5.00	1.50	3.90	0.80
Sunflower meal	9.00	4.20	2.50	1.00
Palmist meal	4.00	4.00	1.20	1.60
Soya pods	1.90	1.90	3.00	3.00
Molasses	2.00	1.00	3.00	3.00
Nutex 68 (Dumoulin Inc)	3.60	3.30	3.30	3.30
Chalk	1.20	1.20	1.60	1.70
Fat	0.50	1.20	1.50	2.20
Rapeseed meal	3.00	2.20	-	-
Rapeseed flour	1.20	1.20	-	-
Soya oil	-	0.50	0.10	-
Minerals & Vitamins	1.20	1.00	1.40	1.30
L-Lysine 50 %	0.40	0.40	0.40	0.40
L-threonine	0.04	0.04	0.08	0.09
DL-methionine	-	-	0.03	0.03
Thr+Met 70/30	-	0.03	-	-
Chemical composition[2]				
DM (%)	88.8	88.8	88.3	88.3
OM (%)	94.1	94.3	93.8	93.9
CP (%)	14.6	14.5	18.1	17.4
ADF (%)	11.9	11.4	9.88	9.75
NDF (%)	22.4	25.4	19.7	20.9
Starch (%)	34.1	30.6	34.2	31.1
Fat (%)	5.53	7.22	6.12	6.97
GE (kcal/kg DM)	4520	4633	4508	4613

[1]GC = gestation, control diet; GWB = gestation, WB diet; LC = lactation, control diet; LWB = lactation, WB diet; [2]DM = analytical dry matter; OM= organic matter; CP = crude protein; GE = gross energy; ADF = acid detergent fiber; NDF = neutral detergent fiber. All diets were analysed for DM (105°C overnight, AOAC 967.03), OM (calcination at 550°C for 12 hours, AOAC 923.03), fat (Soxhlet method, AOAC 920.29), CP (N determination with Kjeltec Analyzer Unit 2300, Foss, Denmark), NDF and ADF (Fibercap system, Foss, Denmark), GE (1241 adiabatic bomb calorimeter, PARR Instrument, USA) and starch content (total starch assay kit, Megazyme, Ireland).

3.4. Sampling of intestinal tissues and rectum content

At days 26 and 27 of age, 16 female piglets having a bodyweight (**BW**) close the the litter mean BW (7.6±0.2 kg for CON group, 7.8±0.2 kg for WB group) were euthanized (8 piglets/treatment, 2 piglets/sow) using an injection of a mix of Xylazine/Zoletil 100 (4 mg of xylazine, 2 mg of zolazepam and 2 mg of tilamine/kg BW) for anaesthesia (Kela SA, Hoogstraten, Belgium; Virbac, Leuven, Belgium), followed by T 61 injection (0.1ml/kg BW) for euthanasia (Intervet Belgium N.V., Brussel, Belgium). The duodenum was collected as the first 15 cm after the pyloric junction, jejunum was collected one meter after this junction and terminal ileum (50cm) was collected before the ileo-caecal junction after clamping to avoid contamination between ileal and caecal contents.The pH of terminal ileum, caecum and colon contents was recorded. Rectum content was collected in sterile tubes and stored at -80°C until further calprotectin analysis.

3.5. Histomorphological analyses

Five-centimetre tissue samples from the duodenum, jejunum and terminal ileum were collected, rinsed with saline solution and dehydrated in 4% formaldehyde for 48h followed by storage in an ethanol 70% solution. Tissues were then embedded in paraffin, and slides cut at 5µm thickness with a microtome using blades Thermo MX35 Ultra (Thermo Fisher Scientific, USA) before haematoxylin-eosin coloration. Villus height and crypt depth were measured at 10-fold magnification using an Olympus BX51 microscope and imaging system (Olympus Corporation, Hamburg, Germany) in 30 well-oriented villi and associated crypts per animal. The images were analysed by image software provided by Olympus.

3.6. Ex vivo experiment and calprotectin concentration

During sampling, terminal ileum tissue was collected, rinsed with culture medium (DMEM, Sigma-Aldrich, St Louis, USA), immediately cut in 6 mm-explants with biopsy punches (Vtrade International, Fernelmont, Belgium) and placed in 6-wells sterile plates containing 3ml of William's culture medium (Sigma-Aldrich, St Louis, USA) supplemented with antibiotics and nutriments as decribed by Lucioli *et al.* (2013). Two modalities were set up on each plate: culture medium was either control (NO-LPS) or supplemented with LPS from *E. coli* O111:B4 (Sigma-Aldrich) at a concentration of 10µg/µl in order to mimic enterotoxigenic *E. coli* (**ETEC**) infections. This B4 strain at that concentration was already used successfully in other experiments (Leonard *et al.*, 2012; Mukhopadhya *et al.*, 2014; Vigors et al., 2016). Explants were incubated during 2 hours at 39°C, 5% CO_2, snap-frozen and stored at -80°C until gene expression analyses. Calprotectin concentration in the rectum content of the piglets was assessed using Porcine Calprotectin *ELISA* Kit (MyBioSource, San Diego, USA) following the manufacturer's recommendation. The quantity of faeces diluted in PBS was 100mg faeces in 1ml of PBS.

3.7. Relative gene expression

RNA was extracted from frozen explants using ReliaPrep™ RNA Tissue Miniprep System kit (Promega, Madison, USA). Concentration and quality of RNA were determined with Nanodrop (Thermo Scientific NanoDrop 2000, USA) and by visualisation on an agarose gel (1%). Subsequently, 3.5µg of RNA were converted to single-stranded cDNA using GoScript™ Reverse Transcription Mix (Promega), following the manufacturer's instructions. Specific regions of cDNA coding for housekeeping genes (PPIA and GAPDH), cytokines and receptors (TNF-α and TLR4) and tight junction proteins (ZO1 and CLDN2) were then amplified with qPCR (StepOne Plus, Thermo Fisher Scientific, USA) by using GoTaq® qPCR Master Mix (Promega). Primers used were either found in literature or designed with primer-BLAST software (NCBI). The list of the primers used is found in Table 9. GAPDH and PPIA were selected as house keeping genes after verifying their stability upon all experimental conditions. QPCR conditions were optimized to obtain primers efficiency values between 90 and 110% (primers concentrations ranged between 300 and 400nM, 40 cycles of qPCR at 60°C) and primers specificity was verified on the melting curves. GAPDH and PPIA were used as reference genes; gene expression was normalized using the $2^{-\Delta\Delta Ct}$ method fixing the value of the CON pigs NO-LPS at 1 for better comparisons.

Table 9. Primers used for qPCR analysis. Genes analyzed are peptidylprolyl isomerase A (PPIA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Tumor necrosis factor α (TNF-α), Toll-like receptor 4 (TLR4), Zonula occludens protein 1 (ZO-1), Claudin 2 (CLDN2). Primers were tested on a pool of cDNA from the explants.

Primer		Sequence (5' → 3')	Reference	Accession number
PPIA	*F*	TAA-CCC-CAC-CGT-CTT-CTT	Dozois et al. (1997)	NM_214353.1
	R	TGC-CAT-CCA-ACC-ACT-CAG		
GAPDH	*F*	CAT-CCA-TGA-CAA-CTT-CGG-CA	Chatelais et al. (2011)	NM_001206359.1
	R	GCA-TGG-ACT-GTG-GTC-ATG-AGT-C		
TNF-α	*F*	ACT-GCA-CTT-CGA-GGT-TAT-CGG	Meissonnier et al. (2008)	NM_214022.1
	R	GGC-GAC-GGG-CTT-ATC-TGA		
TLR-4	*F*	CCT-GAC-AAC-ATC-CCC-ACA-TCA	Designed	NM_001113039
	R	TGC-TCT-GGA-TAG-TGG-TAA-AAG-C		
ZO-1	*F*	TGA-GAG-CCA-ACC-ATG-TCT-TGA-A	Vigors et al. (2016)	XM_021098856
	R	CTC-AGA-CCC-GGC-TCT-CTG-TCT		
CLDN2	*F*	AGG-CCT-CCT-GGG-CTT-CAT	Vigors et al. (2016)	XM_021079578.1
	R	GGA-GTA-GAA-GTC-CCG-CAG-GAT		

4. Results

4.1. Zootechnical parameters

Sows' ingestion (Figure 9) was not affected by the dietary treatment except for the last period, where the ingestion of sows fed the WB diet was significantly reduced compared to the CON (66.2% of the target ingestion reached *vs.* 88.7%, respectively, $p<0.001$). No effect of the maternal treatment on piglets' bodyweight was observed from birth until weaning (Figure 10); an effect of sex was observed ($p=0.017$), male piglets being heavier than females at every time point. The litter size (13±0.82 piglets for CON, 13.25±0.92 piglets for WB $p=0.84$) and number of weaned piglets (8.86±0.77 for CON sows, 9.87±0.61 for WB sows, $p=0.31$) did not differ between both groups. Diet did not impact the changes of sows' backfat thickness at any time point. The same observation was made on the sows' bodyweight gain or loss (data not shown).

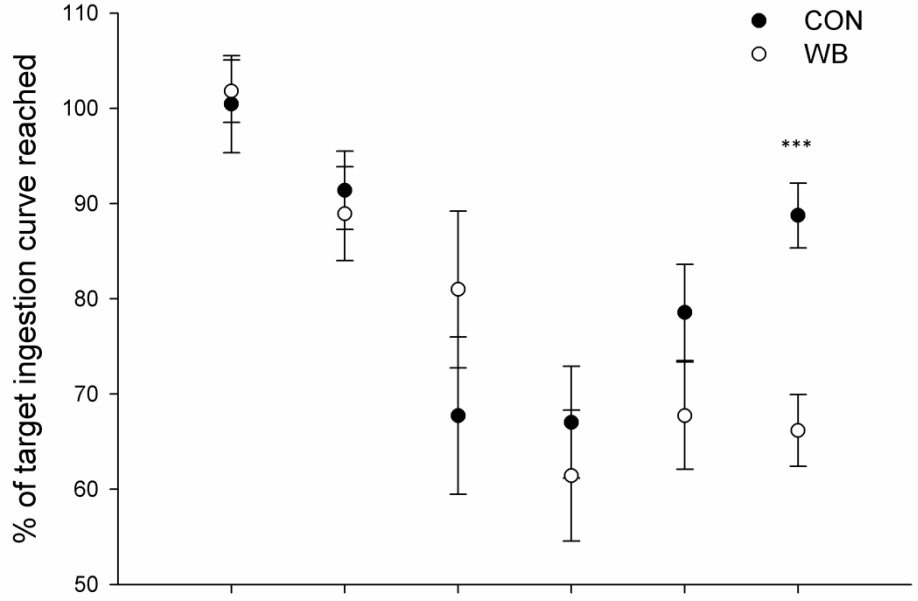

Figure 9. Percentage of target ingestion curve reached for sows in 4-days periods from farrowing until weaning. CON sows (N=7) are represented by the black dot and WB sows (N=8) by the white dot. Values are mean ± SEM. *** represent a p-value<0.001.

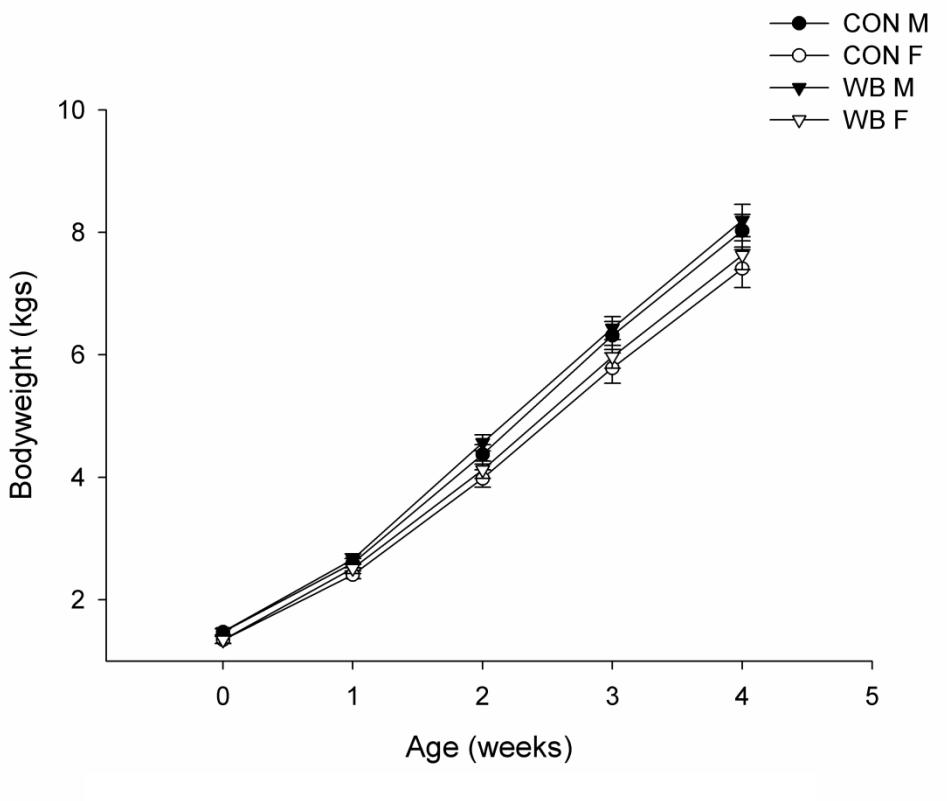

Figure 10. Weekly bodyweight of piglets born from WB and CON sows, from birth (week 0) until weaning (week 4). CON piglets are represented by dots and WB piglets by triangles; black symbols are males and white ones are females. Results are shown as mean ± SEM, n=54 for CON piglets and n=71 for WB piglets

4.2. Milk composition

Protein and fat concentration were impacted by the parity of the sows (p=0.04 and 0.03, respectively, Table 10). Moreover, an interaction between treatment and parity was observed for protein concentration which was translated by a treatment effect for the primiparous sows (9.46±1.75% of protein for CON *vs* 8.84±1.43 % for WB sows). For both protein and lactose concentration, primiparous sows had a higher percentage than multiparous sows (9.11±1.09% of protein for P1 sows *vs* 8.71±0.99% for P≥2 sows; 9.13±0.38% of fat for P1 sows *vs* 8.53±0.32% for P≥2 sows). Lactose percentage was not affected by the parity of the sows but globally by the dietary treatment (p=0.046). This treatment effect was not observed for every time point but a significant difference between treatments was observed during the second week of lactation (4.81±0.04% of lactose for CON sows *vs* 4.89±0.02% for WB sows,

p=0.03). All parameters were impacted by the week of sampling, highlighting the difference between colostrum and milk. Indeed, protein and immunoglobulin contents decreased after one week while fat and lactose concentration increased ($p<0.001$). None of the measured immunoglobulins was impacted by the dietary treatment, while only IgA concentration had significant interactions. Indeed, in the colostrum of sows, a parity effect was observed, P1 sows' colostrum containing lower amounts of IgA than P≥2 sows' colostrum (11.97±0.43 mg/ml and 15.03±1.31mg/ml, respectively).

4.3. Intestinal parameters

At weaning, the pH of the intestinal content of the three intestinal parts (distal ileum: 6.53±0.14 for CON, 6.66±0.17 for WB, caecum: 6.17±0.14 for CON, 6.26±0.32 for WB, colon: 6.22±0.16 for CON, 6.33±0.21 for WB) and the relative lengths of the intestinal tract (1.17±0.14 m/kg BW for CON, 1.13±0.16 m/kg BW for WB) were not affected by the maternal dietary treatment ($p>0.05$). Calprotectin concentrations in the rectal content of the piglets were not affected ($p=0.72$) by the maternal dietary treatment, values reaching 40.67±2.54 ng/ml for the CON piglets *vs* 39.55±0.99 ng/ml for the WB piglets. Villi in the duodenum of WB piglets (Figure 11) were significantly higher (359.4±6.8µm for CON *vs* 455.2±7.0µm for WB, $p<0.05$) while crypts tended ($p<0.10$) to be lower in the jejunum of the WB pigs (Figure 11). The ratio villi/crypt was increased for WB piglets in the duodenum (1.34±0.03 CON vs 2.02±0.13 WB) and jejunum (1.27±0.03 CON *vs* 1.60±0.04 WB). No difference was observed between maternal treatments concerning the ileal parameters.

Table 10. Fat, protein, and lactose percentage, IgA, IgG and IgM concentrations in milk samples collected on a weekly basis after farrowing for piglets born from control (CON) or wheat bran (WB) sows. Values are presented as mean and SEM are given for each period, n=3 or 4 for each parity within a treatment.

Sample	Treatment	Parity	Protein (%)	Fat (%)	Lactose (%)	IgA (mg/ml)	IgG (mg/ml)	IgM (mg/ml)
Colostrum	CON	P1	19.49	6.48	2.60	12.63	68.58	4.99
		P≥2	18.77	6.21	2.60	14.81	59.74	4.73
	WB	P1	18.36	6.63	2.66	11.48	65.69	4.23
		P≥2	20.40	6.08	2.56	15.25	71.31	4.21
	Global SEM		0.30	0.14	0.03	0.81	3.46	0.32
Milk W1[1]	CON	P1	6.10	10.26	4.75	1.84	0.42	0.92
		P≥2	6.22	9.29	4.59	2.30	0.39	1.21
	WB	P1	5.83	10.37	4.71	2.49	0.34	0.95
		P≥2	6.10	8.59	4.77	2.57	0.48	1.35
	Global SEM		0.10	0.51	0.05	0.16	0.05	0.11
Milk W2	CON	P1	5.95	10.93	4.79	2.26	0.27	1.10
		P≥2	6.10	9.02	4.82	2.55	0.32	1.09
	WB	P1	5.43	9.68	4.85	2.36	0.24	0.97
		P≥2	5.80	9.55	4.93	2.96	0.27	1.19
	Global SEM		0.10	0.28	0.02	0.17	0.02	0.07
Milk W3	CON	P1	6.31	10.84	4.81	3.22	0.21	1.01
		P≥2	6.15	8.88	4.84	3.56	0.20	0.95
	WB	P1	5.74	8.31	4.95	3.77	0.16	0.98
		P≥2	5.98	9.40	4.91	3.37	0.16	1.03
	Global SEM		0.08	0.38	0.03	0.23	0.02	0.08
Overall P-values	treatment		0.16	0.23	**<0.05**	0.77	0.52	0.58
	time		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	parity		**0.04**	**0.03**	0.75	0.06	0.82	0.76
	treatment*time		0.48	0.48	0.74	0.68	0.80	0.31
	treatment*parity		**<0.01**	0.16	0.58	0.82	0.28	0.73
	time*parity		0.4	0.63	0.20	**0.03**	0.34	0.19
	treatment*time*parity		0.12	0.06	0.25	**0.04**	0.28	0.90

[1]Milk W1 is milk one week after farrowing, W2= 2 weeks, W3= 3 weeks after farrowing.

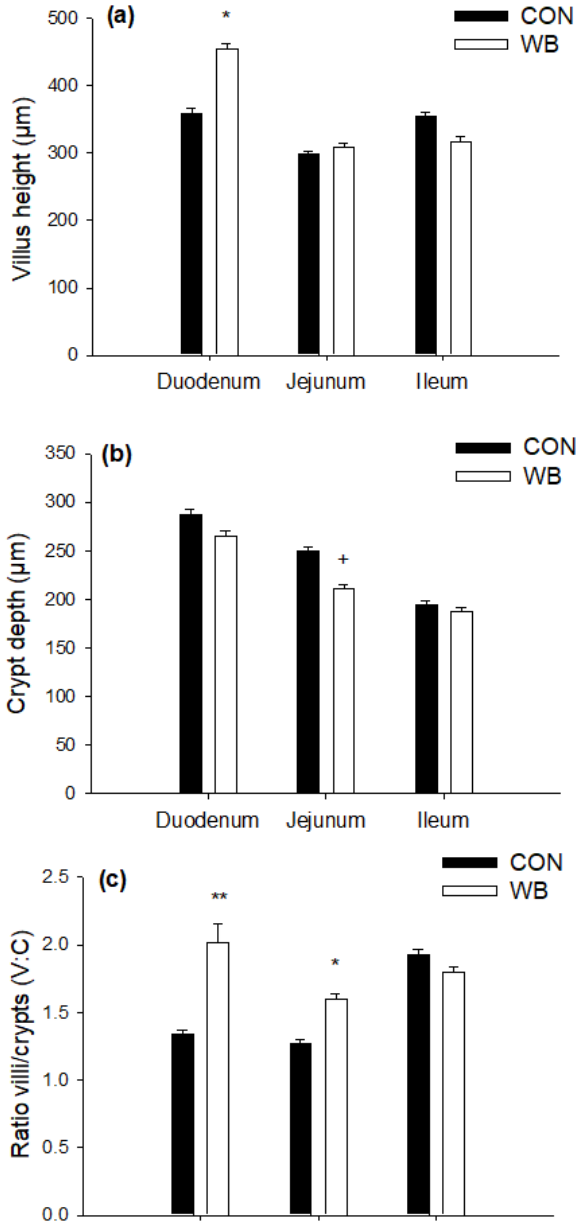

Figure 11. Villus height (a), crypt depth (b) and ratio V:C (c) of piglets botn from CON (black bar, N=8) or WB (grey bars, N=8) sows. Values are mean ± SEM. P-values <0.05 and <0.01 are indicated with the symbols * and **, P<0.10 are indicated with the symbol +.

4.4. Relative gene expression

No impact of LPS or the maternal treatment was observed on piglets' ileum expression of the tested genes (Figure 12).

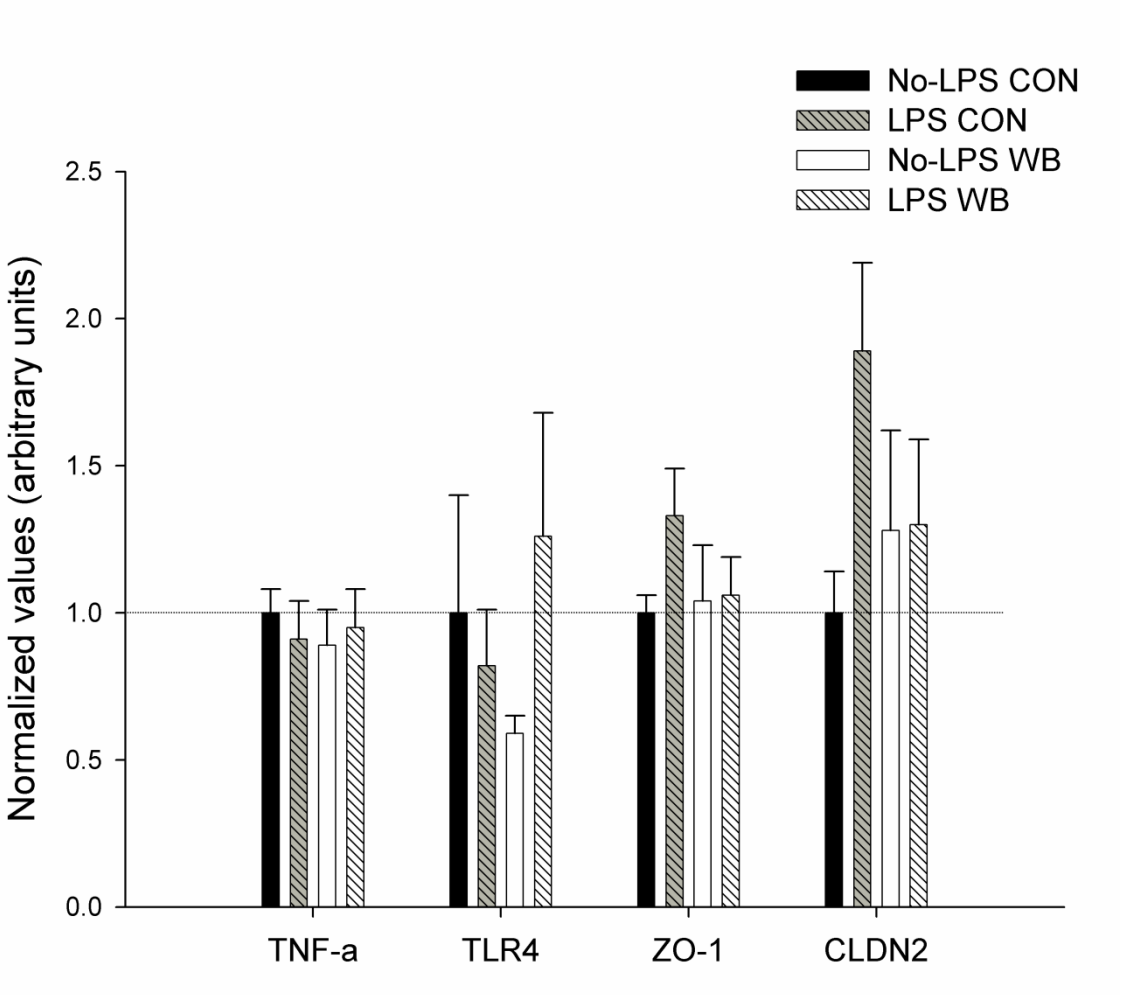

Figure 12. Relative gene expression for TNF-α, TLR4, ZO-1 and CLDN2 in piglets' ileum tissue challenged or not with LPS. No-LPS CON is set to 1 to serve as reference for comparison; n=8 piglets/maternal treatment.

5. Discussion

The aim of this study was to explore effects of feeding a high WB diet to sows on the progeny and in particular to investigate if this maternal diet could impact the performances and intestinal health parameters of the progeny. The hypothesis under this research question is that effects on piglets could be the result of an altered microbiota of sows or of an altered milk composition following the addition of WB. However, only the effect of milk was investigated in this study as microbiota changes had already been studied in Leblois et al. (2017).

5.1. Sows and piglets' performances

Overall performances of the sows were not impacted by the dietary treatment. Indeed, no effect of WB on the backfat or bodyweight changes of the sows was observed, which is in contrast with another study using high fibre diet (Danielsen and Vestergaard, 2001). Litter size and piglets' growth were not affected either, which is in line with other high-fibre diets studies (Loisel et al. 2013; Matte et al. 1994). These equal performances between dietary groups are desirable in order to increase WB proportion in the diet without impairing performances. Sows' feed intake was similar between treatments during the first periods (periods 1-5), while it decreased in the last period of lactation. The explanation for this decrease is unclear, as sows were fed a high WB diet during the last month of gestation and the whole lactation period, so it seems that the drop in intake cannot be ascribed to the bulking of WB.

5.2. Milk composition and gut morphology

The absence of differences in milk composition concerning immunological or nutritional components between treatments can partly explain the equal performances of sows and piglets observed between treatments (weekly bodyweight of the piglets and number of weaned piglets). Indeed, sows' WB diet did not impact colostrum and milk immunoglobulins concentrations, sows providing the same passive immunity to the piglets enhancing their survival until weaning. Interestingly, other studies using bioactive compounds like seaweed (Leonard et al., 2012) or short-chain fructo-oligosaccharides (Le Bourgot et al., 2014) found an increase in colostral IgA, IgG or TGFβ1. Milk protein and fat percentages were not affected by the sows' dietary WB. Hurley (2015) highlighted that protein content is generally not affected by the diet, as observed in this study. However, some studies showed an increase in protein content of the milk of sows fed a yeast-mannan rich diet (Graugnard et al., 2014) or a decrease for sows fed high alfalfa diet (Krogh et al., 2017). The only nutritional component that differed between treatments was lactose, being higher in WB sows' milk than in CON sows at every time point. It can be possible that this higher lactose concentration could have resulted in a higher absorption of glucose and galactose as also the villi/crypts ratio was higher in the duodenum and jejunum and as Heim et al. (2015a) mention the relation between the glucose transporter SGLT1 and villus

height in the ileum of piglets. Moreover, the higher V/C ratio, suggesting a better absorption of nutrients, might also prevent a severe villi shortening at weaning, maybe impacting growth on a long term, which remains to be investigated.

5.3. Intestinal fermentation and intestinal health

In our previous study (Leblois et al., 2017), we showed only small differences in the colonic microbiota of piglets born from WB or CON sows, together with higher acetate and lower butyrate and valerate production in the caecum of piglets born from WB sows. However, no difference in the global short-chain fatty acids production was observed between groups, explaining the similar intestinal pH of the piglets born from the two dietary groups; these results are in line with Paßlack et al. (2015) when feeding sows a 3% inulin diet. Calprotectin, a well-known biomarker in human for inflammatory bowel disease in human faces (D'Haens et al. 2012; Foell et al., 2009) did not show differences in both groups, which suggest no intestinal inflammation for piglets even though the pertinence of calprotectin in pigs still needs to be demonstrated.

The lack of major differences in piglets' microbiota (Leblois et al., 2017) and immunoglobulin content of colostrum might be responsible for the absence of a maternal effect on the gut inflammation, as microbiota plays an important role in the maturation of the immune system. In line with this, Leonard et al. (2012) who supplemented sows with seaweed during lactation, did not observe any maternal effect on the immune response either in the ileal or colonic cultured tissues, except for TNF-α that was more expressed in the ileum of challenged tissues from piglets born from seaweed supplemented sows.

Surprisingly, the LPS challenge did not seem to induce inflammation, as TNF-α concentration did not increase and as the receptor for LPS, TLR-4, was not more expressed in LPS-challenged explants. In contrast, an increased TNF-α expression has been reported when incubating colonic explants with 10µg/ml LPS from B4 *E. coli* strain for 3 hours (Bahar et al. 2016; Mukhopadhya et al. 2014). Another study also determined the effects of LPS from B4 strain 10µg/ml on different genes expression in ileum tissue (Vigors et al., 2016). The authors did not observe any impact of the LPS challenge on TNF-α but an increased expression of other pro-inflammatory cytokines IL-1, IL-6, IL-8 e.g. and a decreased expression of TLR4, ZO-1 and CLDN-2, which was not observed in this study. These 3 studies were performed on explants from already weaned or adult animals, making the comparison difficult as the gut immune system is not as mature at our time point as the other studies. Moreover, Leonard et al. (2012), using biopsies of 26-days old piglets, as in our study, observed an increase of IL-6 and IL-1α in the LPS-challenged explants of ileum and an increase of TNF-α concentration in the ileum of piglets born from seaweed-supplemented sows but not in the ileum of piglets born from control sows.

Thus the lack of inflammation is not clear and for future experiments we might need to adapt the dose of LPS and the duration of incubation.

6. Conclusion

A high WB maternal diet did not impact overall piglets' performances until weaning or intestinal immune parameters. However, there was an impact on the villi/crypt ratio, which might affect performance on a long term. As no detrimental effect was observed on the performances of the sows or the piglets, a higher proportion of WB in sows' gestating (25% of WB) and lactating (14% of WB) diet could be applied, although research on a larger number of animals and until slaughter weight of the pigs might first be recommended.

Acknowledgments

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Declarations of interest: None

5

In vitro characterization of different resistant starch sources

Different sources of resistant starch *in vitro* show contrasting fermentation and SCFA profiles

(based on an abstract submitted for a poster presentation at EAAP 2017, Tallinn)

1. Introduction

As wheat bran only induced slight changes in the microbiota of sows and piglets, a second feed ingredient was selected for the second animal experiment. Resistant starch, considered widely as prebiotic, seemed a good candidate to be included in the diet of sows. The aim of this experiment was to select one source of RS from different purified ingredients based on their fermentation pattern and butyrate production *in vitro*.

2. Materials and methods

An *in vitro* fermentation was performed, following the protocol described by Bindelle et al. (2010). Briefly, five different sources of purified native RS2 (two high amylose maizes, one potato starch and two pea starches) were firstly hydrolyzed in vitro to mimic digestion in the stomach and small intestine with porcine pepsin and pancreatin. Undigested residues were recovered by dialysis membrane (1000kD) and used as substrate for in vitro fermentation. In vitro fermentation was performed by using 5% of sows' faeces in Menke buffer; 30ml of buffer was added to 200mg of substrate in sealed vials and incubated at 39°C for 72h. At 8, 12 and 24h of fermentation, 2ml of fermentation juice was collected and stored at -20°C before SCFA determination by isocratic HPLC using a Waters system fitted by an Aminex HPX-87H column (Bio-Rad, Hecrules, CA, USA) combined with a UV decteror (210nm) and sulfuric acid 5mM flowing at 0.6ml/min for the mobile phase. Gas pressure was measured 2, 5, 8, 12, 16, 20, 24, 48 and 72h after the beginning of the fermentation to allow the determination of fermentation kinetics using the model of Groot et al. (1996). Several parameters were calculated with this model: A (maximal gas volume, ml/g DM), B (time to reach A/2, h), Rmax (maximal fermentation rate, ml/g DM*h) and Tmax (time to reach Rmax). Statistical analyses were performed with the MIXED procedure of SAS, using the substrate as fixed factor.

3. Results and discussion

From Table 11, it is clear that pea starch A (Nastar, Cosucra, Belgium) is the most promising RS from the fermentation kinetics point of view. Indeed, this starch produces the highest gas volume in the shortest time; in comparison, high amylose maize have the lowest gas production and take more time to be fermented; potato starch is in between, having a lower gas production but with an intermediate time to

reach A/2. These results are clearly shown in Figure 13. Giuberti et al. (2013), who also performed *in vitro* fermentation with different sources of either digestible or resistant starch, observed the same gradation for Rmax between pea, high-amylose and potato starches.

Table 11. Fermentation kinetics parameters for the different RS sources fermented *in vitro*. N=3 per substrate. A= maximal gas volume; B= time to reach A/2; Rmax= maximal fermentation rate; Tmax= time to reach Rmax.

Sample	A (ml/g DM)	B (h)	Rmax (ml/g DM*h)	Tmax (h)
Maize A	$220.4^{b} \pm 12.2$	$13.7^{a} \pm 0.4$	$24.2^{b} \pm 2.1$	$12.9^{a} \pm 0.7$
Maize B	$220.1^{b} \pm 13.9$	$13.2^{a} \pm 0.6$	$25.2^{b} \pm 2.3$	$12.4^{a} \pm 0.8$
Pea A	$252.6^{a} \pm 9.0$	$10.3^{c} \pm 0.1$	$48.8^{a} \pm 2.0$	$10.0^{b} \pm 0.1$
Pea B	$221.4^{b} \pm 7.8$	$9.2^{c} \pm 0.2$	$27.8^{b} \pm 1.8$	$8.3^{c} \pm 0.4$
Potato	$212.7^{b} \pm 12.0$	$11.1^{b} \pm 0.7$	$19.7^{c} \pm 1.8$	$9.7^{b} \pm 0.5$

Within a column, values having different superscript letters ($^{a, b, c}$) show significant differences ($p<0.05$)

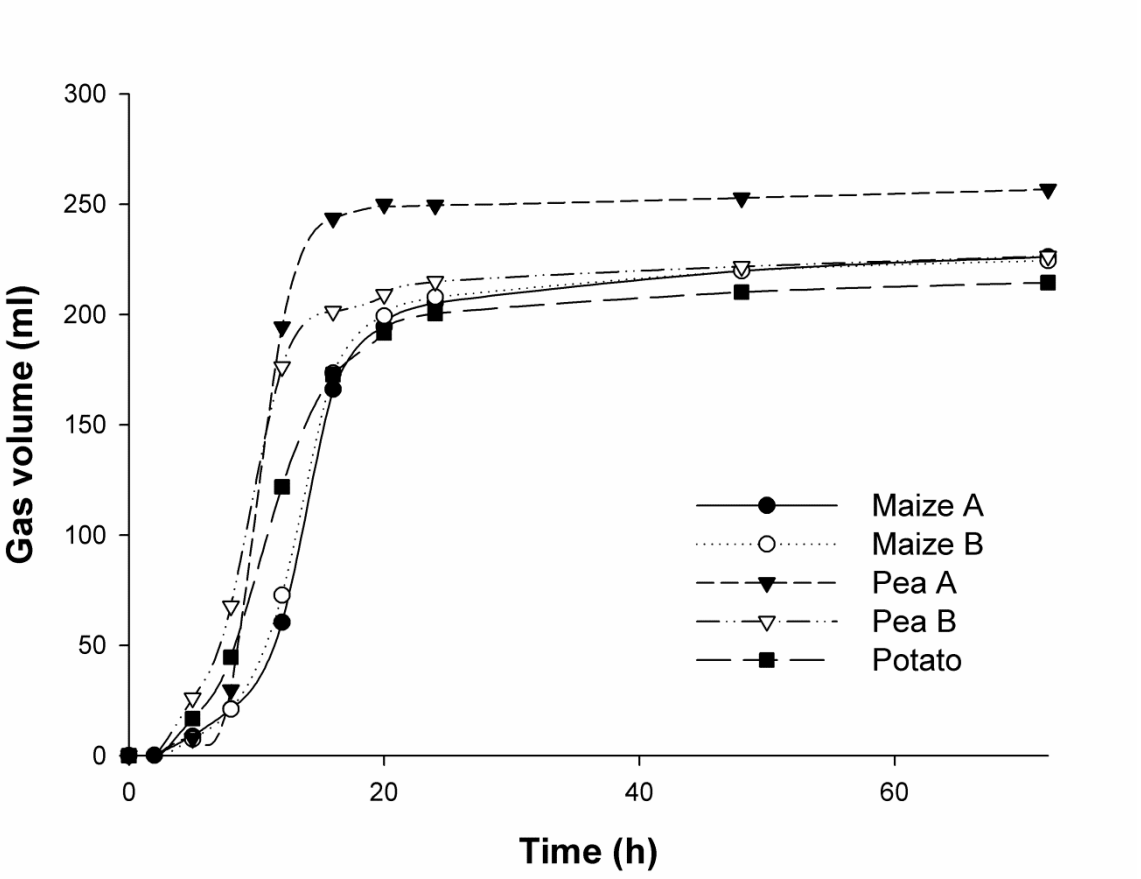

Figure 13. Total gas production curve of the different RS substrates over time.

Total SCFA production and molar ratios of acetic acid, propionic acid and butyric acid were highly ($p<0.05$) impacted by the substrate used (Table **12**). Indeed, total SCFA production at every time point was the highest for Pea starch B, while potato and pea A starches had intermediate values (24h) between pea B and the two high amylose maizes. Molar ratios were also impacted by the substrate, and in particular butyrate was highly influenced, as pea starch A produced the highest level of butyrate at the 3 sampling points, while high amylose starches produced the lower butyrate proportion, as already observed by Giuberti et al. (2013). Acetate and propionate production were also impacted by the substrate, maize starch producing in general more acetate than pea starches, while the opposite effect was observed for propionate production after 8 and 12h of fermentation.

Table 12. Total SCFA production and individual molar ratios of acetate, propionate and butyrate produced by the 5 types of RS at 3 time points.

Time	Ingredient	Sum (mg/ml)	%acetate	%propionate	%butyrate
8h	Maize A	1.7^{d}	72.5^{a}	20.6^{bc}	6.9^{d}
	Maize B	1.7^{d}	70.8^{a}	21.6^{b}	7.6^{d}
	Pea A	2.0^{c}	70.3^{a}	18.3^{c}	11.4^{a}
	Pea B	2.8^{a}	66.7^{b}	23.3^{a}	10.0^{b}
	Potato	2.3^{b}	66.5^{b}	24.8^{a}	8.8^{c}
12h	Maize A	2.6^{i}	73.3^{f}	20.5^{i}	6.2^{i}
	Maize B	2.8^{h}	70.7^{g}	22.7^{g}	6.6^{i}
	Pea A	3.8^{g}	61.0^{i}	17.6^{j}	21.5^{f}
	Pea B	4.3^{f}	64.2^{h}	21.2^{h}	14.6^{g}
	Potato	3.8^{g}	63.9^{hi}	23.6^{f}	12.5^{h}
24h	Maize A	4.6^{w}	68.8^{v}	25.8^{w}	5.3^{y}
	Maize B	4.8^{vw}	66.6^{w}	27.4^{v}	6.0^{y}
	Pea A	4.7^{vw}	60.7^{y}	18.5^{z}	20.8^{v}
	Pea B	5.0^{v}	65.2^{x}	20.8^{y}	14.0^{w}
	Potato	4.9^{v}	63.0^{y}	24.1^{x}	12.9^{x}

Within every time point in the same column, values having a different superscript letter ($^{a-d}$ at 8h; $^{f-j}$ at 12h; $^{v-z}$ at 24h) are significantly different ($p<0.05$).

4. Conclusion

Based on the kinetics of gas production and from the SCFA and more specifically butyrate results, pea starch, and in particular pea starch A (Nastar, Cosucra, Belgium) seemed to be a suitable candidate to be incorporated in the diet of sows in order to modify favourably their microbiota and subsequent butyrate production. These results led to the choice of pea starch for the second animal experiment, as this type of RS was able to ferment rapidly with the highest butyrate production, which was the aim of this *in vitro* experiment. The increased butyrate production might be the result of a higher abundance of butyrate-producing genera that could possibly improve gut health.

6

Feeding sows resistant starch during gestation and lactation impacts their faecal microbiota and milk composition but shows limited effects on their progeny

Article 3 (published in PLOS ONE)

Feeding sows resistant starch during gestation and lactation impacts their faecal microbiota and milk composition but shows limited effects on their progeny

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1. Abstract

Background: Establishment of a beneficial microbiota profile for piglets as early in life as possible is important as it will impact their future health. In the current study, we hypothesized that resistant starch (RS) provided in the maternal diet during gestation and lactation will be fermented in their hindgut, which would favourably modify their milk and/or gut microbiota composition and that it would in turn affect piglets' microbiota profile and their absorptive and immune abilities.

Methods: In this experiment, 33% of pea starch was used in the diet of gestating and lactating sows and compared to control sows. Their faecal microbiota and milk composition were determined and the colonic microbiota, short-chain fatty acids (SCFA) production and gut health related parameters of the piglets were measured two days before weaning. In addition, their overall performances and post-weaning faecal score were also assessed.

Results: The RS diet modulated the faecal microbiota of the sows during gestation, increasing the *Firmicutes*:*Bacteroidetes* ratio and the relative abundance of beneficial genera like *Bifidobacterium* but these differences disappeared during lactation and maternal diets did not impact the colonic microbiota of their progeny. Milk protein concentration decreased with RS diet and lactose concentration increased within the first weeks of lactation while decreased the week before weaning with the RS diet. No effect of the dietary treatment, on piglets' bodyweight or diarrhoea frequency post-weaning was observed. Moreover, the intestinal morphology measured as villus height and crypt depths, and the inflammatory cytokines in the intestine of the piglets were not differentially expressed between maternal treatments. Only zonula occludens 1 (ZO-1) was more expressed in the ileum of piglets born from RS sows, suggesting a better closure of the mucosa tight junctions.

Conclusion: changes in the microbiota transferred from mother to piglets due to the inclusion of RS in the maternal diet are rather limited even though milk composition was affected.

2. Introduction

Post-weaning diarrhoea is one of the major health problems in pig husbandry worldwide. It is characterized by a higher risk of infections and a lower feed intake, due to the conversion from milk to solid feed, which has consequences on the gut morphology like the atrophy of the small intestinal villi and hyperplasia of the crypts (Lallès et al. 2004; Montagne et al. 2007; Hu et al. 2014). Weaning troubles are also accompanied with an impairment of the immune function, a higher permeability of the gut mucosa to antigens and lower brush border enzymes activity (lower lactase

and sucrase activities), lowering the ability of the piglets to digest feed (Le Huërou-Luron 2003; Lallès et al. 2004; Montagne et al. 2007; Gresse et al. 2017).

Feeding strategies to reduce the risk of post-weaning diarrhoea include the use of prebiotics, probiotics and organic acids in newly weaned piglets' diet (Gresse et al. 2017). The mode of action of these feed ingredients relies on their ability to modify favourably the microbiota of the piglets which is very important for their health. Indeed, beneficial bacteria can act as a barrier against pathogens, having the ability to lower the pH of the gastrointestinal tract and produce anti-microbial compounds (Sassone-Corsi & Raffatellu 2015). Microbiota fermenting indigestible carbohydrates produces SCFA that are an important energy source for the animal and butyrate in particular is a gut health-promoting compound acting as the main energy source for colonocytes and exerting anti-inflammatory properties (Guilloteau et al. 2010b). It is thus of interest to modify favourably the microbiota towards fermentative butyrate-producing and anti-pathogenic bacteria.

Different moments in the life time of piglets for the feed additive supplementation are currently envisaged in research. The first strategy to favour beneficial bacteria in the gut early in life is to feed the additives to newly weaned piglets to boost their immunity via the development of a beneficial microbiota at weaning. Another strategy is the use of these additives in the sows' diet in order to promote a rapid colonization of beneficial bacteria and a long-lasting effect for the health of the progeny (Starke et al. 2013; Paßlack et al. 2015; O'Doherty et al. 2017). Several mechanisms are hypothesized concerning the maternal effect. Firstly, acting on the sow's diet relies on the fact that the microbiota triggering the intestinal immune system in piglets will be acquired from the bacteria present in sows' faeces, vagina and in milk (Starke et al. 2013; Paßlack et al. 2015). The purpose then is to modulate the microbiota of the sows to shape a beneficial colonizing microbiota in piglets, improving their immune competence. Secondly, another mechanism that is sought is the modification of the composition of the milk, for nutrients and immunoglobulins (Igs) composition (Krogh et al. 2017), as it has been shown in sows fed a high fibre diet (Loisel et al. 2013) or a diet rich in short-chain fructooligosaccharides (Le Bourgot et al. 2014). As microbiota impacts the development and maturation of the intestinal immune system (Schokker et al. 2014), and as immunoglobulins act as a first passive immunological defence for piglets (Salmon et al. 2009; Theil et al. 2012), modifying one or another of these components, or possibly both together, could promote a healthy gut and prepare the piglet for the weaning period.

Resistant starch is the part of starch that escapes enzymatic digestion in the small intestine and can thus be fermented in the colon of the pig (Haenen et al. 2013; Giuberti et al. 2015). It generally comes in ingredients with high amylose contents. Resistant starch can be classified in 5 categories depending on its chemical and physical properties: RS1 (physically inaccessible starch), RS2 (native resistant starch

granules), RS3 (retrograded starch), RS4 (starch that has been chemically modified) and RS5 (amylose-lipid complex starch) (Giuberti et al. 2015; Yan et al. 2017). As a non-digestible but fermentable dietary component, the inclusion of resistant starch in the diet is expected to reduce the energy content of the diet (Gerrits et al. 2012), potentially reducing performances and/or feed conversion compared to digestible starch. In turn, it should modulate microbiota composition in the distal small intestine and the large intestine of the animals and subsequently impact fermentation end-products. The production of butyrate is usually specifically increased as starch fermentation is known for being butyrogenic (Bindelle et al. 2008; Pieper et al. 2015).

Thus, the purpose of this study was to investigate whether maternal pea starch supplementation could impact the ability of piglets to cope with the weaning period and its associated stresses by comparing the composition of the faecal microbiota and the milk of sows fed two diets contrasting in resistant starch contents. Additionally, the performance, health status and gut immune and morphological status of their progeny was also compared as well as their intestinal microbiota. Pea starch was used as a source of RS because of its ability to produce a high ratio of butyrate during in vitro fermentation (Giuberti et al. 2013); it is considered to be a RS2 type (Themeier et al. 2005; Giuberti et al. 2015) and contains 35% of amylose (information provided by the supplier).

3. Materials and methods

3.1. Animals, diets and housing

All experimental procedures led on sows and piglets were in accordance with European and Belgian regulations concerning laboratory animal welfare. The ethical protocol was reviewed and approved by the Animal Ethical Committee of Liège University (protocol number: 1661). Sows and piglets were housed until weaning at the Walloon Agricultural Research Centre (Gembloux). Landrace sows were inseminated with Piétrain semen and housed in groups on straw litter from one week after artificial insemination (AI) until one week before farrowing. Before the diet change, sows were housed all together in a room that was then divided in two parts to avoid cross contamination after diet change. For farrowing and lactation, they were moved to individual farrowing units, equipped with wood shavings litter, a heat lamp and an extra rear space for sows and piglets accessible by day 5 after delivery. Sows were fed a standard gestation diet until day 88 of gestation, after which they were divided in two dietary groups. The first group (12 sows) received a diet containing 33% of digestible starch (DS diet) and the other group (12 sows) received a diet containing 33% of pea starch (Nastar, Cosucra, Belgium), considered as resistant starch (RS diet). One sow from the RS group had to be removed from the experiment as she had to be treated with antibiotics because of vulva gangrene after delivery. Farrowing was induced by the injection of 2 ml of sodium cloprostenol (92 μg/ml) at

114 days of gestation. Within the DS diet, 4 sows were of 1st parity (P1), 2 sows of 2nd parity (P2), 3 sows of 3rd parity (P3) and 3 sows had a parity higher or equal to 4 (P≥4). Within the RS group, the parities distribution was as follows: 4 P1 sows, 2 P2 sows, 4 P3 sows and 2 P≥4 sows.

Gestation and lactation diets contained 33% of starch, were formulated to be iso-nitrogenous and iso-energetic (net energy) according to NRC requirements (Nutrient Requirements of Swine, 2012). The composition of the diets is shown in Table 1. Between gestation and lactation diets, except for the change of barley into wheat, the same ingredients were used. At weaning (day 28), 44 female piglets (4 piglets/sow) were moved to the Animal Productions Centre in Gembloux and were fed a standard post-weaning diet devoid of antibiotics, prebiotics, probiotics or non-starch polysaccharide (NSP) enzymes. Two littermates were kept together in the same pen and the temperature the day of arrival was maintained at 26°C.

Table 13. Composition of sows' diets during gestation for digestible starch (GDS) and resistant starch (RDS) and during lactation (LDS and LRS) and analyzed chemical composition.

	GDS	**GRS**	**LDS**	**LRS**
Pea Starch	-	33.0	-	33.0
Maize starch	33.0	-	33.0	-
Wheat bran	12.5	12.5	12.4	12.4
Sugar beet pulp	10.1	10.1	6	6
Soya meal	5.1	5.1	13.7	13.7
Sunflower meal	10	10	4.5	4.5
Canola meal	5	5	4	4
Palmist meal	4	4	4	4
Wheat	-	-	3.7	3.7
Biscuit flour	3.5	3.5	3.5	3.5
Soya pods	3	3	3	3
DDGS maize	3	3	3	3
Barley	2.6	2.6	-	-
Maize gluten	2	1.8	1.01	0.81
Soya oil	0.3	1.5	1	2.2
Rapeseed flour	1.5	1.5	1.5	1.5
Molasses	1	-	1	-
Chalk	0.94	0.94	1.58	1.58
Fat	0.79	0.79	1.25	1.25
L-lysine	0.37	0.37	0.26	0.26
Salt	0.36	0.36	0.39	0.39
L-thr	0.08	0.08	0.05	0.05
DL-met	0.07	0.07	0.03	0.03
L-try	0.01	0.01	0.05	0.05
Minerals & Vitamins	0.62	0.62	1.102	1.102
Chemical composition analyzed[1]				
DM (%)	89.56	90.21	89.80	90.42
OM (%)	84.96	85.43	85.11	84.95
CP (%)	15.37	15.78	16.40	15.05
NDF (%)	22.81	18.26	17.86	17.54
ADF (%)	11.84	9.52	8.68	8.18
EE (%)	2.82	4.53	4.59	5.6
GE (kcal/kg DM)	4009	4110	4049	4112
Total starch (%)	34.4	29.5	31.4	32.8
Resistant starch (%)	0.88	5.41	0.55	8.55

[1]DM: dry matter; OM: organic matter; CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre; EE: ether extract, GE: gross energy.

3.2. Zootechnical performances

Bodyweight and backfat thickness (Renco lean-meater® of Secrepro, Québec, Canada) of the sows were recorded at days 80 and 107 of gestation and day 28 of lactation to determine the changes between periods. The duration and the piglet expulsion rate of farrowing were determined by recording the time of birth of every piglet. Piglets were weighed weekly from birth until weaning. After weaning, the diarrhoea status of the piglets was assessed by faecal scoring for 15 days, using a scale going from 0 to 4 (0=hard pellet, 1= soft dry pellet, 2= soft shaped wet pellet, 3= unshaped soft pellet, 4= watery). This score was given individually and a mean was calculated for the pen. Piglets were considered to have diarrhoea when the score was 3 or 4. The presence of diarrhoea (score of 3 or 4) was assigned to a "1" value while the absence of diarrhoea (score of 0, 1 or 2) was assigned to a "0" value. The occurrence of diarrhoea was then calculated. As daily recording did not lead to data normality, 3-days data were averaged grouped for analysis. Diarrhoea occurrence was then calculated as the percentage of piglets having a score of 3 or 4 in each pen (0, 50 or 100%) over 3-days periods. The average daily gain (ADG) during the post-weaning period (2 weeks after weaning) was measured by weighing the piglets on a weekly basis.

3.3. Feed chemical analyses

Diets were analysed for organic matter (ashing at 550°C for 6h, AOAC 923.03), dry matter (drying at 105°C for 24 h, AOAC 967.03), crude protein (N determination with Kjeltec Analyzer Unit 2300, Foss, Denmark, CP = N×6.25), ether extract (Soxhlet method using ether petroleum, AOAC 920.29), ash-corrected neutral and acid detergent fiber (Fibercap system, Foss, Denmark, Van Soest et al. 1991 [25]) and gross energy (1241 adiabatic bomb calorimeter, PARR Instrument, USA). Starch (total and resistant) was analysed with the enzymatic kit D-Glucose-HK (Megazyme, USA), quantifying glucose concentration after hydrolysis of starch with pancreatic amylase.

3.4. Milk

Colostrum was collected within one hour after the birth of the first piglet. Milk samples were collected after the intramuscular injection of 2ml of oxytocin on a weekly basis. Samples were filtered on sterile medical gauze and stored at -20°C until analysis. Protein, lactose and fat contents in milk and colostrum were determined by Fourier transform infrared spectroscopy on a Standard Lactoscope FT-MIR automatic (Delta Instruments, Drachten, The Netherlands). The predictive models provided by the manufacturer were originally designed for cow milk and were consequently adapted for sow milk by a slope and bias correction using a reference set of sow milk for which composition was analysed by standard wet chemistry methods. The R^2 for each parameter reached 0.99. The IgG and IgA concentrations

of colostrum were determined using specific anti-pig antibodies by ELISA (Bethyl Laboratories, Montgomery, USA and R&D Systems, Oxon, UK), following the manufacturer's recommendations. The plates were read at 450nm on a 96-wells plate reader (Stat-fax 2100, awareness technology Inc, Palm City, USA).

3.5. Sampling of intestinal tissues and contents

Faeces were collected directly from the rectum of the sows in sterile bags at day 106 of gestation and day 15 of lactation. They were immediately snap-frozen in liquid nitrogen and stored at -80°C until DNA extraction. Two days before weaning (day 26 of lactation), 16 female piglets (8 DS, 8 RS, 1 piglet/sow) were euthanized by injection of a mix of Xylazine/Zoletil 100 (4 mg of xylazine, 2 mg of zolazepam and 2 mg of tilamine/kg BW) for anaesthesia followed by bleeding. Content from the caecum and the colon as well as tissue from the ileum and colon of the piglets were collected, snap-frozen and stored at -80°C until further analysis. Tissue samples of 5 cm were collected from the duodenum, jejunum and terminal ileum, rinsed with a saline solution and dehydrated in 4% formol prior to long term storage in 70% ethanol. Tissues were then embedded in paraffin, cut with a microtome using Thermo MX35 Ultra blades (Thermo Fisher Scientific, USA) and stained with haematoxylin and eosin. Villus heights and crypt depths were measured on 30 well-oriented couples villus/crypt per animal by 10-fold magnification microscopy (Olympus BX51 Olympus, Japan).

3.6. Microbiota composition

DNA was extracted from the sows' faeces (10 sows/treatment) and piglets colon contents (8 piglets/treatment) using Qiagen QIAamp Stool Minkit (Qiagen, Hilden, Germany), following the manufacturer's instructions but adding two bead beating steps (FastPrep-24, MP Biomedicals, Illkirsh, France). Quality of DNA was checked on 1% agarose gel and the DNA concentration was assessed by a Nanodrop (Thermo Scientific NanoDrop 2000, USA). DNA was stored at -20°C until sequencing. Sequencing was performed by DNAVision (Gosselies, Belgium), using the Illumina MiSeq (2 × 300nt) and after amplifying, purifying and tagging the hypervariable regions V3-V4 (Forward primer: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′ and reverse primer: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′) following the 16 S Metagenomic Sequencing Library Preparation protocol (Part # 15044223 Rev. B) from Illumina.

3.7. Bioinformatics analysis

Raw sequences of 16S rRNA were assigned to each sample, quality checked and trimmed using Basespace default parameters (Illumina). Sequences were assigned to 97% ID OTUs by comparison to the Greengenes reference database 13.8 using the QIIME (Quantitative Insights Into Microbial Ecology) 1.9.0 software. Since samples contained variable number of sequences (62529 ± 4522 for sows, 94139 ± 13830 for piglets), diversity analyses were carried out on samples rarefied at the same sequencing depth (15973 for sows and 53592 for piglets) to avoid bias in sequencing depth between samples. The Beta_diversity_through_plots.py script was used to assess differences in bacterial communities between groups of samples. Beta diversity was visualized using un-weighed and weighed UniFrac distances with Principal Coordinate Analysis (PCoA). The compare_categories.py script, which applied the adonis method on the previously obtained dissimilarity matrices, was used to determine whether communities differed significantly between groups of samples. In addition, the PERMANOVA procedure was performed by period using R studio software (R Studio, Boston, USA), considering the parity (primiparous *vs* multiparous) and the treatment as factors. Multiple_rarefactions.py and alpha_diversity.py scripts were applied to compute alpha diversity metrics, which evaluated diversity within a sample and generated rarefaction curves. Raw sequences have been uploaded in the European Nucleotide Archive database under the project number PRJEB25722.

3.8. Short-chain fatty acids determination and calprotectin concentration

Faeces, colon and caecum contents (day 26) were analysed by isocratic HPLC as detailed in Leblois et al. (2017) [26]. Briefly, 1g of sample was diluted in 5g of ultrapure water to reach a 6-fold dilution. Samples were then vortexed for 1 minute to ensure a good solubility and homogeneity of the samples. Aliquots of 2 ml were then centrifuged at 13,000 g, acidified with H_2SO_4 and filtered at 0.22 µm. Samples were then analysed for SCFA concentration on a Waters system equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) combined with a UV detector (210nm) at 58°C. The mobile phase was H_2SO_4 5mM. Peaks were integrated with Empower 3 software (Waters, Milford, USA) after the encoding of a standard curve. Results are expressed as $mmol.g^{-1}$ and molar ratios, taking into account the initial dilution. Calprotectin concentration in the colon contents of the piglets was assessed using Porcine Calprotectin *ELISA* Kit (MyBioSource, San Diego, USA) following the manufacturer's recommendations. Absorbance was measured at 450nm.

3.9. Gene expression analysis

RNA was extracted from frozen ileum and colon tissue (day 26) using ReliaPrep™ RNA Tissue Miniprep System kit (Promega, Madison, USA). RNA concentration was determined with a Nanodrop (Thermo Scientific NanoDrop 2000, USA) and integrity was checked on a 1% agarose gel. Then, 2 µg of RNA were converted to single-stranded cDNA using GoScript™ Reverse Transcription Mix (Promega, Madison, USA), following the manufacturer's instructions. Specific regions of cDNA coding for housekeeping genes - *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *beta actin* (*ACTB*)- , tight junction proteins -*zonula occludens-protein 1* (*ZO-1*) and *Occludin* (*OCLN*)- and proteins involved in the inflammatory response - *tumor necrosis factor alpha* (*TNF-α*), *interleukin 6* (*IL-6*), *nuclear factor kappa B* (*NF-κB*), *transforming growth factor beta* (*TGFβ*), *interferon gamma* (*IFNγ*), *interleukin 1 beta* (*IL-1β*) and *interleukin 10* (*IL-10*)- were then amplified with qPCR (StepOne Plus, Thermo Fisher Scientific, USA) using SYBR Premix Ex Taq II (TakaraBio). Primers and their reference are shown in Table 2. QPCR conditions were optimized to obtain primer efficiency values between 90 and 110% (denaturation at 95°C for 5s, annealing at 60°C for 30s and elongation at 72°C for 30s) and primers specificity was verified through melting curves. GAPDH and ACTB were used as reference genes and were selected after verification of their stability for all experimental conditions.; gene expression was normalized using the $2^{-\Delta\Delta Ct}$ method setting the value of the DS pigs to 1 to allow comparisons.

3.10. Statistical analyses

All statistical analyses were performed on SAS 9.2 (SAS Inc, USA). Gut morphology was analysed with the NESTED procedure of SAS, with the treatment as fixed class factor and piglet as random factor; piglets' bodyweight until weaning was determined with the mixed procedure of SAS, time being a repeated effect, sow(treatment) being a random effect and treatment and parity being fixed factors. Milk composition and sows' performances were analysed with the repeated MIXED procedure of SAS; treatment and parity were used as fixed effects and time was used as a repeated factor. Duration of farrowing and expulsion rate were determined with the MIXED procedure of SAS, using parity and treatment as fixed effects. Calprotectin, gene expression and SCFA were analysed with the MIXED procedure of SAS, using the maternal treatment as fixed factor. Diarrhoea score, piglets' bodyweight and average daily gain (ADG) post-weaning were analysed with the repeated MIXED procedure of SAS, with time as repeated factor and maternal treatment as fixed effect. Microbiota results were analysed with the non-parametric Kruskall-Wallis test added by Benjamini-Hochberg correction; maternal treatment was included in this test as fixed effect. Pearson's correlations were determined between the abundance of *Lactobacillus* and of other genera with a relative abundance of >1% of the total microbiota using the proc CORR of SAS. P-values

<0.05 were considered as significant. For microbiota analysis, a 0.05<p<0.10 was considered as a trend.

Table 14. Primers used for gene expression analysis.

Primer		Sequence (5'->3')	Reference	Accession number
ACTB	*F*	GGA-CTT-CGA-GCA-GGA-GAT-GG	Dozois et al. (1997)	XM_021086047
	R	GCA-CCG-TGT-TGG-CGT-AGA-GG		
GAPDH	*F*	CAT-CCA-TGA-CAA-CTT-CGG-CA	Chatelais et al. (2011)	NM_001206359.1
	R	GCA-TGG-ACT-GTG-GTC-ATG-AGT-C		
TNF-α	*F*	ACT-GCA-CTT-CGA-GGT-TAT-CGG	Meissonnier et al. (2008)	NM_214022.1
	R	GGC-GAC-GGG-CTT-ATC-TGA		
IL-6	*F*	AGA-CAA-AGC-CAC-CAC-CCC-TAA	Vigors et al. (2016)	NM_214399
	R	CTC-GTT-CTG-TGA-CTG-CAG-CTT-ATC		
TGFβ	*F*	GAA-GCG-CAT-CGA-GGC-CAT-TC	Meurens et al. (2009)	NM_214015
	R	GGC-TCC-GGT-TCG-ACA-CTT-TC		
IFNγ	*F*	TGG-TAG-CTC-TGG-GAA-ACT-GAA-TG	Royaee et al. (2004)	NM_213948
	R	GGC-TTT-GCG-CTG-GAT-CTG		
NF-κB	*F*	CCT-CCA-CAA-GGC-AGC-AAA-TAG	Alassane-kpembi et al. (2017)	ENSSSCT00000033438
	R	TCC-ACA-CCG-CTG-TCA-CAG-A		
IL-1β	*F*	ATG-CTG-AAG-GCT-CTC-CAC-CTC	Gourbeyre et al. (2015)	NM_214055
	R	TTG-TTG-CTA-TCA-TCT-CCT-TGC-AC		
IL-10	*F*	CTG-CCT-CCC-ACT-TTC-TCT-TG	Feng et al. (2015)	NM_214041
	R	TCA-AAG-GGG-CTC-CCT-AGT-TT		
ZO-1	*F*	TGA-GAG-CCA-ACC-ATG-TCT-TGA-A	Vigors et al. (2016)	XM_021098856
	R	CTC-AGA-CCC-GGC-TCT-CTG-TCT		
OCLN	*F*	CTA-CTC-GTC-CAA-CGG-GAA-AG	Chen et al. (2013)	NP_001157119.1
	R	ACG-CCT-CCA-AGT-TAC-CAC-TG		

4. Results

4.1. Zootechnical parameters

No differences between treatments were observed concerning the duration (250 ± 27 min for DS vs 243 ± 53 min for RS, p=0.95) or expulsion rate (one piglet every 19±2 and 20±5 min for the DS and RS groups, p=0.63) of farrowing. Changes in bodyweight and backfat thickness between periods were not affected by the dietary treatment either (S1 Table). The survival of piglets until weaning was not affected by the treatment (86.5% of piglets for DS vs 84.7% for RS, p=0.82). No impact of the maternal treatment or the sex was observed for piglets' bodyweight until weaning (S1 Fig). None of these parameters were impacted by the parity of the sow.

4.2. Colostrum and milk

Globally, lower milk protein concentrations were observed in the RS sows than in the DS group (p=0.02). An interaction between the treatment and sow parity was observed (see Table 15, $p<0.05$), showing that for P3 sows, the milk protein percentage was lower at every time point for RS sows (Table 16). Fat percentage was not affected by the RS diet, but an interaction between parity and time was significant ($p<0.05$). Only in colostrum, parity influenced the fat concentration as the colostrum of first parity sows was richer in fat and then gradually decreased with parity. Milk lactose percentage was not impacted by the treatment (p=0.09) and a significant interaction between time and treatment was observed (p=0.01). For colostrum and milk samples collected during week 1, RS sows secreted more lactose ($p<0.05$) in their milk while this concentration was lower during the last week of gestation ($p<0.05$). Milk composition changed with time, as protein concentration decreased over time while lactose and fat increased (Table 16).

Table 15. P-values of the treatment, time, parity and interactions for protein, fat and lactose content of the milk.

	Protein	**Fat**	**Lactose**
Treatment	**0.02**	0.74	0.14
Time	**<0.001**	**<0.001**	**<0.001**
Parity	0.37	0.07	0.30
Treatment*Time	0.11	0.11	**0.01**
Treatment*parity	**0.02**	0.13	0.13
Time*parity	0.43	**0.02**	0.59
Treatment*Time*parity	0.43	0.32	0.93

Table 16. Composition (protein, fat and lactose) of colostrum and milk of sows fed digestible starch (DS) and resistant starch (RS) in function of parity. N=12 for the DS group and N=11 for the RS group after because of removal of one sow from the experiment.

			Time			
	Diet	**Parity**	**Colostrum**	**Week 1**	**Week 2**	**Week 3**
Protein (%)	DS	P1	18.46	6.25	5.63	5.59
		P2	18.61	6.32	5.39	5.16
		P3	19.76	6.42	6.29	6.78
		P≥4	18.83	7.52	5.88	5.60
	RS	P1	17.35	6.09	5.76	5.73
		P2	18.36	6.01	5.80	5.97
		P3	17.33	5.79	5.41	5.69
		P≥4	17.99	5.85	5.89	5.63
	Global SEM		0.33	0.16	0.1	0.14
Fat (%)	DS	P1	9.22	8.90	9.29	8.57
		P2	7.82	8.37	8.72	7.14
		P3	7.78	8.43	9.87	10.46
		P≥4	5.34	8.96	8.46	7.62
	RS	P1	8.76	8.69	9.70	9.94
		P2	8.39	9.17	8.38	9.54
		P3	6.94	8.03	7.55	9.01
		P≥4	6.01	8.95	8.48	8.74
	Global SEM		0.37	0.31	0.28	0.35
Lactose (%)	DS	P1	2.81	4.75	4.96	4.91
		P2	2.75	4.92	5.13	5.17
		P3	2.67	4.75	4.86	4.99
		P≥4	2.69	4.88	5.02	5.18
	RS	P1	2.88	4.89	4.89	4.86
		P2	2.83	4.88	5.13	4.86
		P3	3.07	4.98	5.10	4.95
		P≥4	2.97	4.98	5.02	5.03
	Global SEM		0.05	0.03	0.04	0.03

Immunoglobulin G, the most abundant Ig in colostrum, was not affected by the dietary treatment (55.2±4.17 mg/ml for DS group *vs* 52.1±2.69 mg/ml for the RS group, p=0.80) but the parity tended (p=0.06) to affect the IgG concentration, milk of P≥4 sows having higher IgG concentration than P1 and P3 sows (66.23±5.97 *vs* 48.93±4.41 and 48.58±2.21 mg/ml, respectively). IgA concentration in colostrum was not affected by the treatment (p=0.07) but an effect of the parity was observed (p<0.01), IgA concentration being the lowest for the first parity (Figure 14)

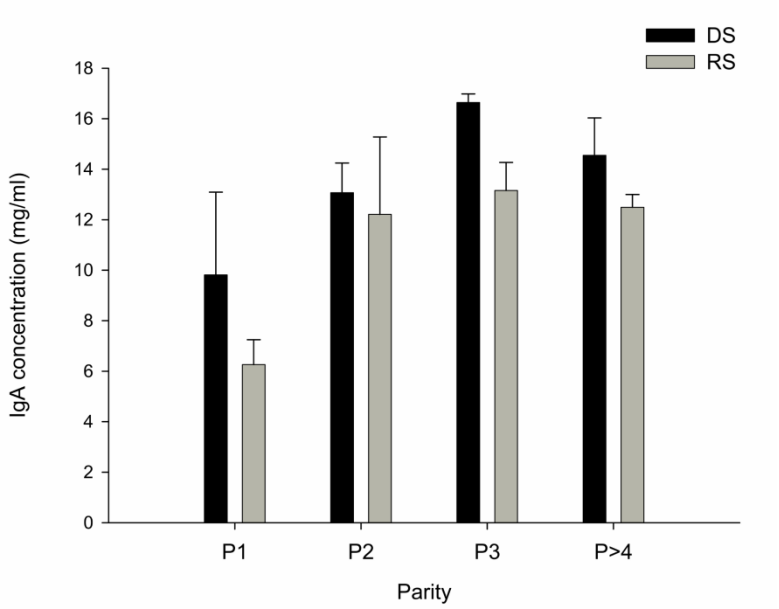

Figure 14. IgA concentration (mg/ml) in colostrum of sow. DS sows are represented with the black bar (N=12) and RS sows with grey bars (N=11) sows. Results are expressed as mean+SEM.

4.3. Microbiota of sows' faeces

Microbiota composition of the sows was determined during gestation and lactation; the effects of treatment and period were assessed. The number of observed OTUs and bacterial diversity (Shannon and Chao1 indexes) did not show any differences between treatments within each period. However, a different microbiota composition was observed between periods as seen by the PCoA discriminating gestation and lactation (Figure 15). Between gestation and lactation, a trend almost reached significance for a higher bacterial diversity during gestation as represented by the Shannon index (Gestation=7.8±0.3 and lactation=7.6±0.3, p=0.06). Although bacterial diversity did not differ between treatments (Shannon index, P >0.10), the composition of the microbiota was affected during gestation as shown by the PCoA analysis (Figure 16). This clustering disappeared during the lactation period (S2 Fig). Statistical analyses for beta diversity showed the effect of the diet during gestation (p=0.001) but no more during lactation (p=0.56), while the parity did not seem to impact beta diversity, even though a trend was present (p=0.09 during gestation and p=0.07 during lactation).

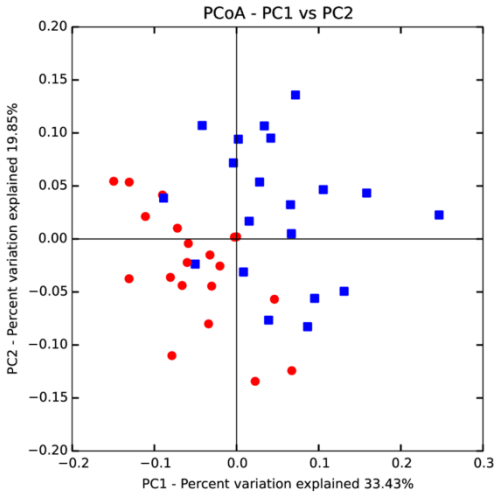

Figure 15. PCoA discriminating periods. Individual red dots are the fecal samples of sows during gestation (N=20) while blue squares are individual fecal samples of lactating sows (N=20).

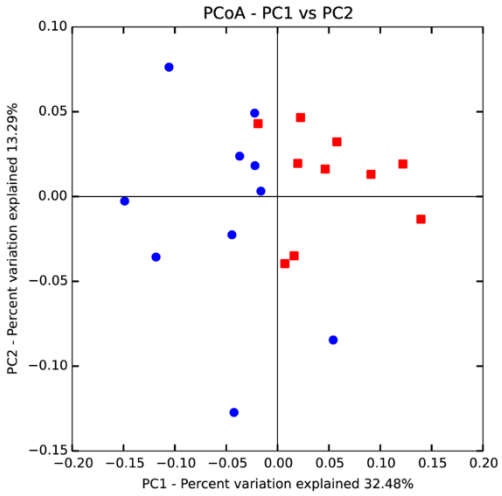

Figure 16. PCoA discriminating dietary treatments during gestation. Red squares represent the faecal microbiota composition of sows fed DS during gestation (N=10) while blue dots represent microbiota of sows fed RS diet (N=10).

The clustering between treatments during gestation translated differences in microbiota composition both at the phylum and genus levels. At the phylum level, the most abundant phyla during both periods for the two groups were *Firmicutes*, *Bacteroidetes* and Spirochaetes. Differences in microbial composition between treatments were observed during gestation as *Firmicutes* ($p<0.01$, FDR < 0.05) and Euryarchaeota ($p<0.05$, FDR=0.05) proportions in the faecal microbiota of RS sows increased while *Bacteroidetes* ($p<0.01$, FDR<0.05), Spirochaetes ($P<0.01$, FDR<0.05) and Tenericutes ($P<0.01$, FDR=0.17) relative abundances decreased compared to the DS treatment. During lactation, only the minor Phylum Lentisphaerae ($p<0.01$, FDR=0.12) proportion increased in the RS group compared to the DS group. The ratio *Firmicutes*:*Bacteroidetes* was impacted by the dietary treatment during gestation (1.59±0.07 for DS vs 2.11±0.15 for the RS sows, p=0.005) while no effect of the dietary treatment was observed during lactation (2.36±0.32 for DS vs 2.43±0.17 for RS sows, p=0.84).

At the genus level (Table 17), the major differences in sows' faecal microbiota composition between treatments also appeared during gestation. The most abundant genera were an unclassified Ruminococcaceae, *Prevotella*, unclassified Bacteroidales and Clostridiales, and Treponema. Within these major components of faecal microbiota, the unclassified Ruminococcaceae was increased ($p<0.05$) and Treponema was decreased ($p<0.05$) significantly during gestation in the RS group; this difference disappeared during lactation. Twelve other genera differed ($p<0.05$) between gestation while only 6 genera differed during lactation; the relative

abundances of *Bifidobacterium*, *Coprococcus*, an Unclassified *Clostridiales* OTU2, *Sharpea*, *Methanobrevibacter* and an unclassified *Peptostreptococcaceae* relative abundances were increased ($p<0.05$) in the faeces of sows fed RS during lactation. While *Oscillospira* decreased during lactation. An unclassified *Clostridiaceae*, SMB53 and *Turicibacter* increased both during gestation and lactation. It is worth noting that the proportion of *Lactobacilli* jumped from a mean of 2.38±0.42% during gestation to 11.73±1.50% during lactation, but this difference could not be attributed to the drop of one particular genus as the Pearson correlation analysis did not reveal absolute r-values higher than 0.6 (data not shown).

Table 17. Relative abundances of the phyla and genera in sows' faeces. Only genera present at >0.01% in the faecal microbiota of the sows fed either digestible starch (DS, n=10) or resistant starch (RS, n=10) -based diets during gestation and lactation were considered.

Genus	Gestation					Lactation				
	DS	**RS**	**P**	**FDR**	**SEM**	**DS**	**RS**	**P**	**FDR**	**SEM**
Actinobacteria	1.78	2.14	0.08	NS	0.22	1.88	1.9	NS	NS	0.25
Bifidobacterium	0.92	1.36	**0.02**	NS	0.21	1.18	1.15	NS	NS	0.23
Bacteroidetes	34.27	29.19	**<0.01**	**0.03**	0.96	28.69	27.08	NS	NS	1.13
Prevotella	12.11	8.8	NS	NS	0.9	10.32	10.39	NS	NS	0.91
Unclassified_ *Bacteroidales*	10.75	8.88	NS	NS	0.62	8.54	7	NS	NS	0.58
Unclassified_S24-7	4.06	4.99	NS	NS	0.52	2.9	3.39	NS	NS	0.26
Unclassified_RF16	1.53	0.8	**0.01**	NS	0.16	0.98	0.91	NS	NS	0.11
Unclassified_ p-2534-18B5	1.44	1.97	0.07	NS	0.16	1.3	1.39	NS	NS	0.16
CF231	1.29	0.9	0.07	NS	0.11	1.18	1.32	NS	NS	0.14
Euryarchaeota	0.17	0.51	0.05	0.05	0.07	0.16	0.2	NS	NS	0.03
Unclassified_R4-45B	0.15	0.12	NS	NS	0.01	0.05	0.13	**0.02**	NS	0.02
Firmicutes	53.25	59.86	**0.04**	**0.04**	1.15	62.24	63.7	NS	NS	1.27
Unclassified_ *Ruminococcaceae*	17.75	20.68	**0.02**	NS	0.59	17.1	17.27	NS	NS	0.78
Unclassified_ *Clostridiales* OTU1	7.76	8.54	NS	NS	0.28	7.21	8.17	NS	NS	0.28
Unclassified_ *Lachnospiraceae*	3.71	3.48	NS	NS	0.15	3.92	3.28	0.06	NS	0.14
Phascolarctobacterium	2.9	2.52	NS	NS	0.22	2.24	2.15	NS	NS	0.12
Unclassified_ *Christensenellaceae*	2.79	3.3	NS	NS	0.36	2.5	2.73	NS	NS	0.32
Streptococcus	2.74	2.03	NS	NS	0.83	1.58	3.37	NS	NS	0.58
Oscillospira	2.24	1.92	NS	NS	0.1	2.05	1.78	**0.04**	NS	0.07
Lactobacillus	2.03	2.73	NS	NS	0.42	13.11	10.35	NS	NS	1.5
Unclassified_ *Clostridiaceae*	1.22	1.84	**0.02**	NS	0.11	1.59	2.07	**0.02**	NS	0.12
Coprococcus	0.83	1.22	**0.02**	NS	0.08	1.28	1.4	NS	NS	0.09
Unclassified_ *Clostridiales* OTU2	0.49	0.79	**0.01**	NS	0.05	0.5	0.56	NS	NS	0.03
SMB53	0.49	0.79	**0.05**	NS	0.07	1.05	1.55	**<0.005**	NS	0.08
Turicibacter	0.33	1.1	**<0.005**	NS	0.12	0.65	1.29	**0.01**	NS	0.12
Sharpea	0.21	0.79	**0.03**	NS	0.15	0.05	0.11	NS	NS	0.03
Methanobrevibacter	0.12	0.46	**0.01**	NS	0.06	0.13	0.17	NS	NS	0.03
Helicobacter	0.12	0.06	**0.04**	NS	0.02	0.03	0.05	NS	NS	0.01

Continuation of **Table 17.**

Genus	Gestation					Lactation				
	DS	**RS**	**P**	**FDR**	**SEM**	**DS**	**RS**	**P**	**FDR**	**SEM**
Proteobacteria	2.92	2.29	NS	NS	0.24	1.46	1.75	NS	NS	0.13
Campylobacter	0.97	0.67	0.07	NS	0.08	0.31	0.34	NS	NS	0.04
Unclassified_ *Peptostreptococcaceae*	0.12	0.2	**0.02**	NS	0.01	0.23	0.33	**0.05**	NS	0.03
Spirochaetes	5.25	3.6	**0.03**	**<0.01**	0.28	3.96	3.5	NS	NS	0.26
Treponema	4.2	3.1	**0.01**	NS	0.23	3.25	2.72	NS	NS	0.22
Sphaerochaeta	1.05	0.5	**<0.005**	NS	0.1	0.71	0.78	NS	NS	0.08
Tenericutes	0.35	0.24	**0.04**	NS	0.03	0.38	0.35	NS	NS	0.03
Anaeroplasma	0.18	0.09	0.08	NS	0.02	0.13	0.12	NS	NS	0.01
L7A E11	0.11	0.19	0.07	NS	0.02	0.06	0.08	NS	NS	0.01

4.4. Microbiota of the colonic content of piglets at 26 days of age

The main abundant Phyla in the colon of piglets before weaning were *Firmicutes* (44.2±2.9%), *Bacteroidetes* (39.6±1.8%), *Proteobacteria* (6.9±1.0%) and *Fusobacteria* (5.7±1.9%). They were not impacted by the maternal dietary treatment, neither was the ratio between *Firmicutes* and *Bacteroidetes* (1.35±0.16 for DS piglets; 1.02±0.16 for RS piglets). At the genus level, the microbiota was mainly composed of *Prevotella*, unclassified *Ruminococcaceae*, *Lactobacillus* and *Bacteroides* (see Table 18). None of the 10 most abundant genera in the colon of the piglets were significantly affected by the maternal diet, while only genera present at a lower relative abundance than 1% of the total microbiota showed a trend ($p<0.10$), including *Veillonella*, unclassified *Clostridiales*, *Pasteurellaceae* and *Dethiosulfovibrionaceae* and *Brachyspira*.

4.5. SCFA, calprotectin concentration in digesta and gut morphology

Total content and molar ratios of individual SCFA and branched-chain fatty acids (BCFA) were not affected by the dietary treatment neither in the faeces of sows nor in the intestinal contents of piglets (S2 and S3 Tables). Calprotectin concentration in the colon of piglets did not differ ($p=0.85$) either (39.03 ± 2.56 and 38.45 ± 1.58 pg/ml for piglets born from DS and RS sows, respectively). Maternal dietary treatment had no effect on villus height, crypt depth and the villi/crypts ratio (V:C), either in the duodenum, the jejunum or the ileum of the piglets (S4 Table).

Table 18. Relative abundances in piglets' colonic contents (N=7 for DS piglets, N=8 for RS piglets). Results showed are only for the top 10 genera and the genera with p<0.10 and relative abundance >0.01%.

Genus	DS	RS	P	FDR	SEM
Bacteroidetes	41.48	37.88	NS	NS	1.76
Prevotella	19.39	14.68	NS	NS	1.92
Bacteroides	5.44	8.25	NS	NS	1.24
Unclassified_*Bacteroidales* OTU1	4.51	4.86	NS	NS	0.54
Unclassified_S24-7	4.34	3.43	NS	NS	0.59
Unclassified_*Bacteroidales* OTU2	2.84	2.91	NS	NS	0.45
Firmicutes	42.73	45.42	NS	NS	2.88
Unclassified_*Ruminococcaceae*	12.19	13.43	NS	NS	1.38
Lactobacillus	5.55	3.94	NS	NS	1.02
Unclassified_*Clostridiales* OTU1	3.18	6.46	NS	NS	1.18
Phascolarctobacterium	2.95	3.71	NS	NS	0.25
Oscillospira	2.37	2.75	NS	NS	0.27
Veillonella	1.09	0.44	0.08	NS	0.20
Unclassified_*Clostridiales* OTU2	0.03	0.01	0.06	NS	0.00
Fusobacteria	4.91	6.37	NS	NS	1.94
Fusobacterium	4.91	6.37	NS	NS	1.94
Proteobacteria	7.89	6.12	NS	NS	1.03
Unclassified_*Enterobacteriaceae*	3.21	1.92	NS	NS	0.74
Unclassified_*Pasteurellaceae*	0.01	0.03	0.08	NS	0.01
Spirochaetes	0.75	1.46	NS	NS	0.33
Brachyspira	0.01	0.00	0.06	NS	0.00
Synergistetes	0.12	0.57	NS	NS	0.22
Pyramidobacter	0.11	0.55	NS	NS	0.21
Unclassified_*Dethiosulfovibrionaceae*	0.00	0.01	0.07	NS	0.00

4.6. Gene expression

In the ileum and colon of piglets, no differences were observed for cytokines involved in inflammatory processes, but an effect of the maternal diet was observed on tight junction protein expression. Indeed, *ZO-1* was more expressed in the ileum of piglets born from RS mothers (Table 19). *OCLN* tended to be more expressed in the ileum of RS piglets without reaching significance (p=0.08).

Table 19. Relative gene expression in the ileum and colon of piglets at weaning. The $2^{-\Delta\Delta Ct}$ value of DS piglets is set at for each gene 1 to allow comparisons.

Gene	Ileum				Colon			
	DS (n=7)	RS (n=8)	SEM	P	DS (n=8)	RS (n=8)	SEM	P
TNF-α	1.00	0.91	0.07	NS	1.00	1.02	0.16	NS
IL-6	1.00	0.95	0.21	NS	1.00	2.55	1.04	NS
NFκB	1.00	0.99	0.03	NS	1.00	1.06	0.06	NS
TGFβ	1.00	0.91	0.04	NS	1.00	1.00	0.11	NS
IFNγ	1.00	0.56	0.17	NS	1.00	0.83	0.34	NS
IL-1β	1.00	0.84	0.21	NS	1.00	0.52	0.32	NS
IL-10	1.00	1.11	0.23	NS	1.00	2.08	0.66	NS
ZO-1	1.00	1.16	0.03	**0.02**	1.00	1.23	0.08	NS
OCLN	1.00	1.38	0.09	0.08	1.00	0.80	0.20	NS

4.7. Performances of piglets after weaning

The maternal treatment did not affect the ADG of the piglets (82.07±15.43g/day and 70.48±13.71g/day for the DS and RS piglets respectively during the first week post-weaning, 167.49±20.13g/day and 206.30±21.31g/day for the DS and RS pigs respectively during the second week post-weaning). Bodyweight of the piglets was not affected by the maternal dietary treatment either (6.71±0.31kg and 6.66±0.30kg for DS and RS pigs one week after weaning and 7.88±0.38kg and 8.35±0.36kg for DS and RS pigs 2 weeks after weaning, P for the treatment=0.98).

A time effect ($p<0.001$) and an interaction between time and treatment ($p=0.005$) was observed for the faecal scoring practised after weaning (Figure 17). On day 7, RS piglets had a lower score than DS piglets while they had a higher score on day 13, without reaching significance ($p<0.10$).The diarrhoea occurrence was calculated on 3-days intervals. The diarrhoea occurrence increased from period 1 to 2 and from period 4 to 5 ($p<0.001$, S3 Fig).

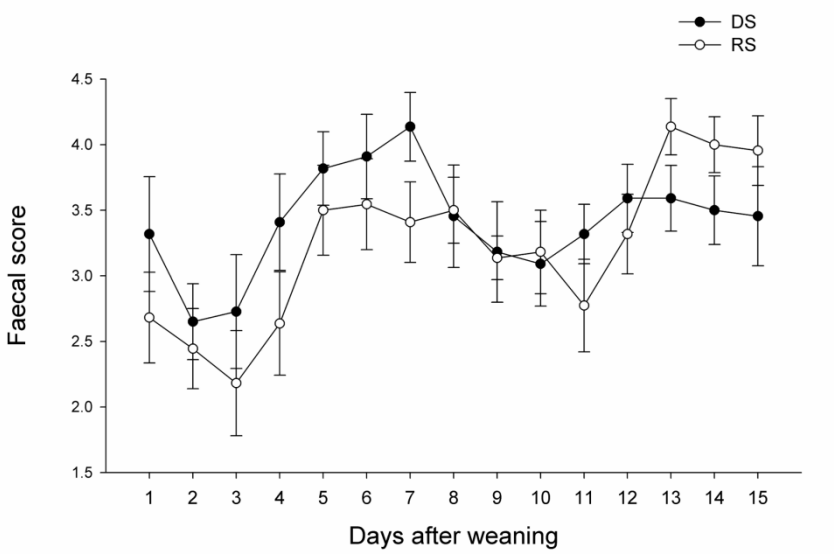

Figure 17. Piglets' faecal score during 2 weeks post-weaning. Score was assessed daily for 15 days.

5. Discussion

The main objective of this study was to investigate the maternal effect of a diet rich in pea starch as a source of RS on the intestinal microbiota and gut health-related parameters of the progeny. The hypothesis was that including resistant starch in the diet of the sows during gestation and lactation would favourably modify their milk and/or microbiota composition and that it would in turn affect piglets' microbiota profile and their absorptive and immune abilities.

The unaffected growth performances of sows and piglets observed in our study is desirable as inclusion of RS, lower in energy content than its digestible counterpart, should not impair the performances of the animal. Yan *et al.* (2017) fed sows high amylose maize (65% during gestation, 60% during lactation) and observed a lower birthweight for piglets born from high amylose sows. However, these piglets were able to catch up during lactation thanks to a higher fat content of the milk. In our study, no impact on the milk fat was observed. A reason for this discrepancy between the present study and Yan *et al.*'s (2017) may reside in the fact that different breeds were used as breed can impact fat concentration (Farmer et al. 2004) and that the amount of RS incorporation in the diet differed. In our study, parity as only factor did not affect the milk fat percentage but the interaction between parity and time was

significant (p=0.02), showing that the colostrum of gilts (parity 1) contained more fat than other parities.

Even though no difference in fat content was observed, other nutritional components of milk were affected by the sows' diet. In particular, a decrease in protein concentration was observed for the RS sows compared to the DS group, together with a higher concentration of lactose in colostrum and milk collected one week after farrowing, while the opposite was observed during the last week of lactation. The lower milk protein concentration could be attributed to a slightly lower analysed protein content of the RS lactation diet. The discrepancy between our study and Loisel *et al.* (2013), who did not observe any increase in lactose concentration in milk during the whole lactation period after feeding sows a high fibre gestation diet, can be explained by the fact that the type of supplementation given to sows differentially affects lactose concentration (Hurley 2015). The increase in lactose concentration in RS milk in the beginning of lactation was probably too small to result in bodyweight difference for the piglets or to affect gut morphology. However, as the milk yield was not measured in this study, it cannot be excluded that DS sows had a higher milk yield, compensating the richer milk of RS sows. In the future, analysing the composition of milk oligosaccharides would be interesting, as oligosaccharides are considered as prebiotics, shaping the gut microbial communities of the piglets and are present in 29 forms in sows' milk (Tao et al. 2010).

Microbiota results showed that in the faeces of the sows, more genera differed between dietary treatments during gestation than during lactation, as already observed by Leblois *et al.* (2017) when feeding sows a high wheat bran diet. During gestation, even if the bacterial diversity and richness were not affected by the diet, we observed a clustering per dietary treatment on the PCoA graph that can be explained by differences both at the phylum and genus levels. Interestingly, during gestation, the RS group had a higher *Firmicutes* to *Bacteroidetes* ratio. As an increased *Firmicutes* proportion is usually related to a higher extraction of energy from the diet (Maga et al. 2012) and an increase in *Bacteroidetes* in humans has been associated with weight loss (Turnbaugh & Gordon 2009); a higher ratio *Firmicutes:Bacteroidetes* would be thus desired in animal production. However, in the short term, the altered ratio observed during gestation did not lead to any bodyweight gain differences between the two groups of sows. In humans however, Martinez *et al.* (2010) observed decreased abundance of *Firmicutes* and increased *Bacteroidetes*, hence a decreased *Firmicutes:Bacteroidetes* ratio, when adding chemically modified RS4 in the diet. Surprisingly, this was not observed for native RS starch granules (RS2) supplementation.

In agreement with the study of Sun et al. (2015) who fed growing pigs raw potato starch and analysed the microbiota in the proximal colon, our study also showed an increase in the abundance of *Turicibacter* and *Coprococcus* and a decrease in Treponema and *Oscillospira* relative abundances in sows' faeces. Interestingly, *Turicibacter* has been reported to be related to host gut immune status as this genus decreased or disappeared in immunodeficient animals (Allen et al. 2015). In addition,

the increase in the beneficial genus *Bifidobacterium* due to RS observed during gestation is in line with other studies (Bird et al. 2007; Martinez et al. 2010). Therefore, the increase in *Bifidobacterium* and *Turicibacter* observed in the current study migh suggest a better gut health. In contrast with Bird et al. (2007) and Haenen et al. (2013), no effect on *Lactobacillus* relative abundance in sows' faeces was observed. Unfortunately, those microbial changes were not transferred to the offspring. It is noteworthy that effects and relative abundances of the genera differ between studies, the main reasons residing in the RS types used, breeds, environment, age, physiological stage, part of the gut studied, choice of hypervariable region for sequencing, and DNA extraction protocols/kits.

While a treatment effect was observed for the sow's faecal microbiota during gestation, these differences disappeared during lactation. Even though ADF and NDF differences between DS and RS diets existed only during gestation, we assume that the microbiota difference is mainly due to the RS difference, for the following reasons. Firstly, RS is more extensively fermented than cellulose and hemicellulose (represented by ADF and NDF, Bindelle et al. 2008). Secondly, the observed genera-changes due to the RS treatment (for which ADF and NDF fractions were lower than in DS diet) during gestation are in line with other studies feeding pigs with RS (Haenen et al. 2013; Sun et al. 2015) and are oriented to fermentative-type bacteria (increased *Firmicutes* and *Ruminococcaceae*). Therefore, we assume the observed changes during gestation can be attributed to the RS rather than the difference in hemicellulose and cellulose content.

The lack of differences in sow's faecal microbiota between treatments during lactation is difficult to explain. However, this is in line with our previous study on wheat bran (Leblois et al., 2017) for which microbiota changes occurred during gestation when feeding sows diets containing the same ADF content but variable amounts of NDF (22% *vs* 25%) and wheat bran (0 *vs* 24%). Hence, it cannot be excluded that the hemicellulose difference existing during only gestation could as well have interfered with the absence of microbial changes during lactation. On the other hand, physiological and environmental changes that the sows face at farrowing and the stresses they encounter throughout the lactation period (manipulation of piglets, milking of the sows) might have such an impact on the microbiota that they can mask the effects of the dietary treatment. Indeed, Paßlack et al. (2015) have already shown a more important time effect (gestation/farrowing/lactation) on the bacterial composition of sows' faeces than inulin effect.

The microbiota changes occurring around farrowing and lactation were probably responsible for the absence of difference between the microbiota of piglets born from DS and RS sows. The similar microbiota between piglets was reflected by the same total and individual SCFA production in the caecum and colon of the piglets. Piglets' microbiota composition considerably differed from the microbiota of the sows, which is in agreement with Leblois *et al.* (2017) and resides in the fact that microbiota still did not reach a stable community that can only be achieved after weaning, maturation and introduction of solid feed. Moreover, piglets' microbiota is not only acquired

from bacteria present in sows' faeces, but also from bacteria present in sows' vaginal tract, milk and in the environment and is unstable in the neonatal gut until reaching a climax community with aging (Bian et al. 2016). It can thus be suggested that changes induced in sows' faecal microbiota induce limited alterations in the colonic microbiota of the piglet.

The immune competence of piglets relies both on the microbial colonization (Schokker et al. 2014) and on the passive immunity acquired from the mother at birth via immunoglobulins transmitted in the colostrum (Theil et al. 2012). Using seaweed extract, Leonard *et al.* (2012) observed an increased concentration of IgG in colostrum of supplemented sows. In another study (Loisel et al. 2013), feeding sows a high fibre diet during gestation decreased IgA concentration 24h after parturition. In our study, IgA concentration in colostrum of RS sows showed a trend ($p=0.07$) for a decrease, while IgG concentration was not affected. A lower concentration of IgA in sows' colostrum would be undesirable as IgA contributes to the passive immunity of the piglets.

The effect of the maternal supplementation with pea starch on the piglets was limited, as the milk composition was barely affected and as the microbiota of piglets was not affected by the maternal treatment. It is then likely that the performances and immune competence of the piglets remained unaffected as observed by similar litter bodyweight gains and percentage of weaned piglets for both treatments. As important as colostrum composition, the microbiota is crucial for the maturation of the gut immune system and has been shown to be the most important factor in the development of the intestinal immune system; moreover, different diets and environments inducing differences in microbiota have been shown to lead to differential immune cells development (Everaert et al. 2017). As no difference in microbiota composition was observed in those piglets raised in the same environment, it seems logical that the immune parameters of the piglets were not affected by the maternal treatment, as determined by the gene expression analysis. In contrast, Heim et al. (2015b) showed that seaweed-derived polysaccharides in the diet of gestating and lactating sows impacted the expression of inflammatory cytokines (higher expression of *IFNγ*, *IL-1*, *TGFβ1* and *TNFα* and lower expression of *IL-10* and *IL-6* in the ileum tissue) of piglets.

However, the protein *ZO-1* was more expressed in the ileum of RS piglets, while OCLN showed a trend ($p=0.08$) for a higher expression. *ZO-1* is called a "plaque protein" and is involved in the strengthening of tight junction proteins by interacting with claudins that are involved in the closure of the gut membrane and also with junctional adhesion molecule Jam-A that is involved in the reduction of intestinal permeability (Ulluwishewa et al. 2011). A higher expression of *ZO-1* is thus beneficial for piglets as this will induce a stronger closure of the gut epithelium and a lower chance of translocation for pathogens, as tight junction proteins are responsible for paracellular permeability (Chen et al. 2013). It is then likely than RS piglets would be less sensitive to pathogens at weaning, together with a lower passage for water loss causing diarrhoea. However, no significant differences on the faecal

score or diarrhoea occurrence during the 2-weeks post-weaning period were observed for piglets born from DS and RS sows, and bodyweight gain or ADG were not affected either.

Thus, using pea starch in sows' diets is not detrimental on piglets' health but to obtain more conclusive results, it may be required to record faecal consistency for a longer period and to collect samples of intestinal tissues and contents further along during the post-weaning phase. In conclusion, the induced microbiota changes due to the diet of the sow did not affect the microbiota of piglets at weaning. However, milk composition can be affected by the inclusion of resistant starch in the diet of sows. Furthermore, the performances of the animals were not impacted by this supplementation and only minor effects of the tight junctions of piglets' intestine were observed.

Acknowledgments

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Appendix 2: Supplementary material article 3

Feeding sows resistant starch during gestation and lactation impacts their faecal microbiota and milk composition but shows limited effects on their progeny

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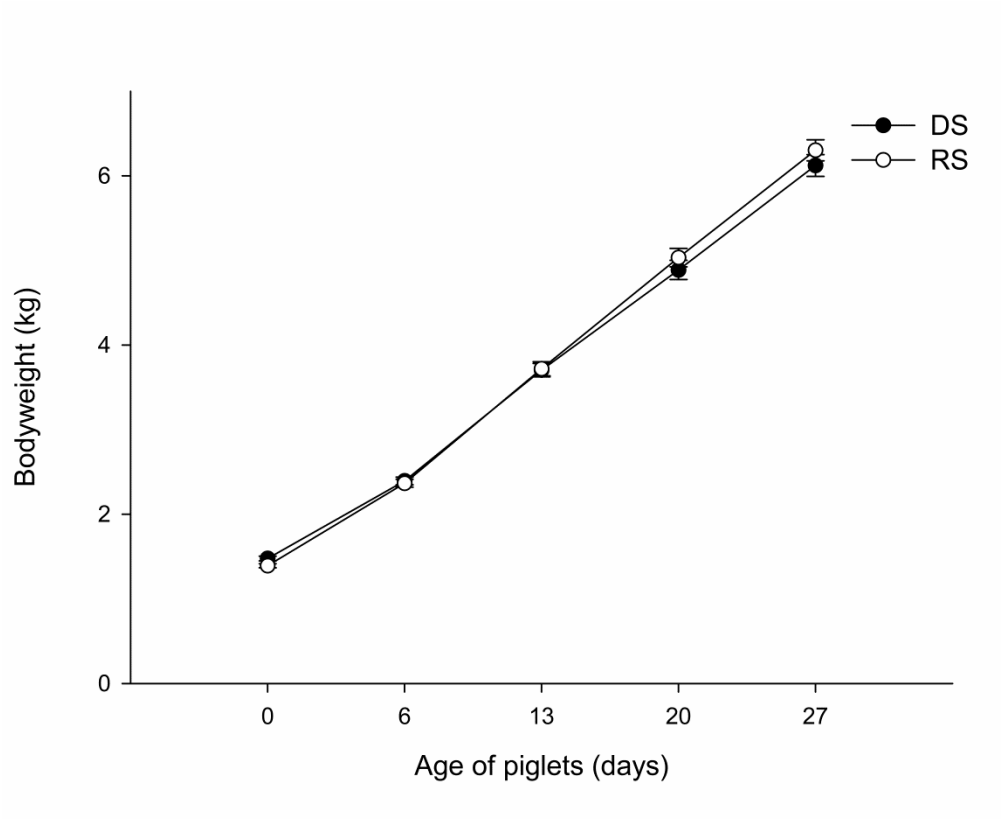
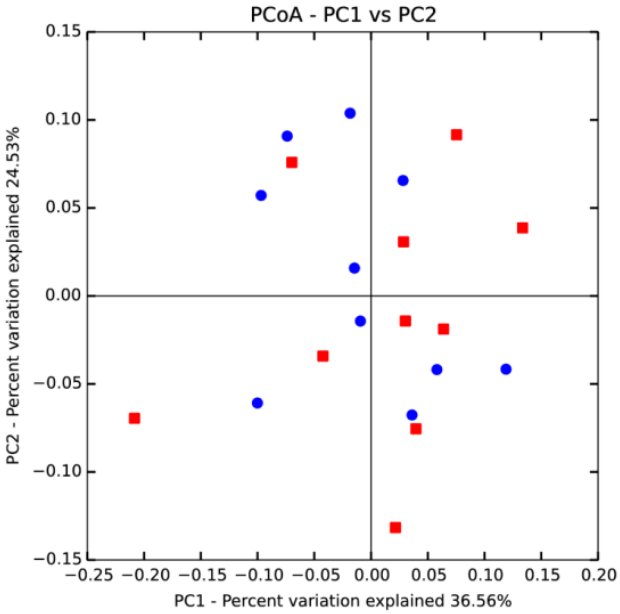
S1 Table. Bodyweight and backfat changes of the sows between periods.

	Period	DS	RS	SEM	P-value
Bodyweight changes (%)	Day 80- day 104	4.04	4.14	0.59	0.94
	Day 104 - weaning	-19.91	-20.01	0.96	0.96
Backfat changes (%)	Day 80- day 104	-1.99	-0.41	1.69	0.65
	Day 104 - weaning	-24.02	-27.06	2.68	0.58



S1 Fig. Piglets' bodyweight from birth until weaning for maternal DS and RS treatments.



S2 Fig. PCoA discriminating dietary treatments during lactation. Red squares represent the faecal microbiota composition of sows fed DS during gestation (N=10) while blue dots represent microbiota of sows fed RS diet (N=10).

S2 Table. Total SCFA and molar ratios of individual SCFA and BCFA in piglets' caecum and colon contents.

	RS	DS	SEM	P value
Sum (mmol/g)	10.55	8.89	0.88	0.35
% acetic acid	58.29	60.41	0.99	0.29
% propionic acid	21.86	21.96	0.54	0.93
% butyric acid	10.66	9.88	0.4	0.35

S3 Table. Total SCFA and molar ratios of individual SCFA and BCFA in piglets' caecum and colon contents

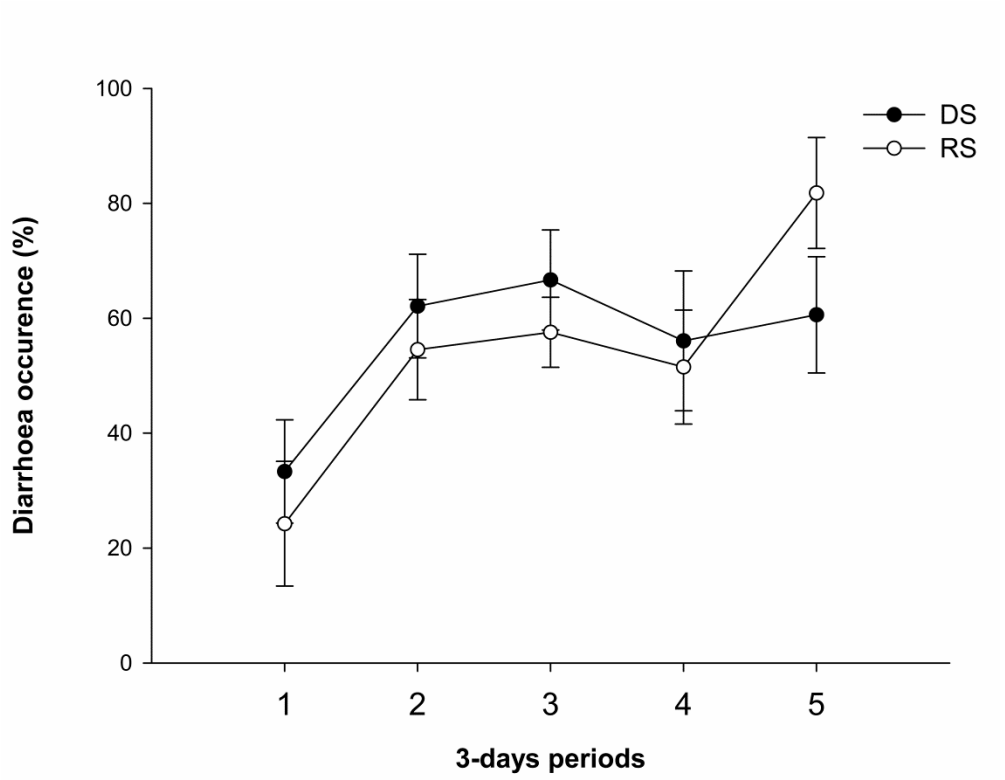
	Caecum			Colon		
	DS	RS	p-values	DS	RS	p-values
Sum (mg/g)	11.0±1.5	8.7±0.9	NS	5.90 ± 1.02	4.82 ± 0.66	NS
Acetate (%)	61.9±2.0	59.3±2.3	NS	53.74 ± 2.95	52.12 ± 2.39	NS
Propionate (%)	20.7±1.0	21.7±1.2	NS	21.52 ± 1.12	23.17 ± 1.88	NS
Butyrate (%)	8.5±0.8	10.5±0.9	NS	10.18 ± 1.76	10.16 ± 1.78	NS
Isobutyrate (%)	2.3±0.1	2.1±0.4	NS	3.83 ± 1.53	2.23 ± 0.69	NS
Isovalerate (%)	1.7±0.2	2.0±0.2	NS	2.8 ± 0.75	2.28 ± 0.60	NS
Valerate (%)	4.1±0.4	3.5±0.8	NS	3.13 ± 0.71	2.55 ± 0.91	NS

S4 Table. Gut morphology (villus height, crypt depth, villus/crypt ratio) in the duodenum, jejunum and ileum of 26-days old piglets.

Villus height (µm)	DS	RS	SEM	P treament	P individual
Duodenum	400.9	397.6	4.35	0.87	<.0001
Jejunum	366.4	406.6	4.43	0.16	<.0001
Ileum	364.8	360.4	4.07	0.87	<.0001
N=240/treatment					

Crypt depth (µm)	DS	RS	SEM	P treament	P individual
Duodenum	313.2	340.3	3.77	0.32	<.0001
Jejunum	289.1	298.2	3.18	0.72	<.0001
Ileum	250.1	222.5	2.67	0.14	<.0001
N=240/treatment					

Ratio V:C (µm)	DS	RS	SEM	P treament	P individual
Duodenum	1.36	1.27	0.02	0.55	<.0001
Jejunum	1.31	1.54	0.04	0.31	<.0001
Ileum	1.53	1.71	0.02	0.26	<.0001
N=240/treatment					



S3 Fig. Diarrhoea occurence for weaned piglets during 15 days, divided in 3-days periods.

7

Maternal dietary resistant starch has lasting effects on their progeny's gut health when challenged with a high fat diet

Following the short-term experiment that aimed at unravelling the effects of introducing RS in the diet of pregnant and lactating sows on the gut health of the progeny at weaning, a long term experiment was led. This experiment aimed at investigating whether the maternal diet supplementation could alter the metabolism and microbiota of their progeny submitted to a high fat challenge in the later life. Below is a small paper concerning this topic but some other results are still waited to complete the discussion and to submit the paper in a journal.

Maternal dietary resistant starch has long lasting effects on their progeny's gut health when challenged with high fat diet

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1. Introduction

Obesity is a metabolic trouble that affected 650 million of adults in 2016 and is related to several health disorders; the first symptom of obesity is overweight, as individuals are considered as obese when BMI>30 (World Health Organization). Moreover, obesity is related to low grade body inflammation represented by an increased expression of IL1β, TNF-α, MCP-1 and IL-6 in muscle, liver and adipose tissue (Cani & Delzenne 2009). In order to investigate the possible dietary solutions to counteract the rise of obesity, animals are most often used as models for humans and in particular rodents were historically the preferred model due to their low cost, ease of handling and short experimental time (Guilloteau et al. 2010a; Gonzalez et al. 2015). Alternatively, the pig is more and more used due to its similar gastrointestinal tract physiology, omnivorous diet and microbiota (Guilloteau et al. 2010a; Heinritz et al. 2013). Possessing similar microbiota is of importance as microbiota plays several roles and is involved in obesity. The roles of the gut microbiota include the extraction of energy from undigested carbohydrates by production of short-chain fatty acids (SCFA) after fermentation that are absorbed by the host, the maintenance of the barrier integrity of the gut by increasing the mucus layer thickness and the settling of the gut immune system (Sommer & Bäckhed 2013). Microbiota has been shown to be involved in the aetiology of obesity as faecal transplantation of obese mice microbiota to germ free (GF) mice led to the appearance of the obese phenotype of these mice (Turnbaugh et al. 2006). In case of obesity, the ratio between *Firmicutes* and *Bacteroidetes* is increased, translating a higher energy extraction from the diet (Maga et al. 2012). Moreover, the abundance of some beneficial bacterial genera, including *Bifidobacterium*, is decreased in case of obesity (Cani & Delzenne 2009). As this genus is involved in the strengthening of the epithelial barrier, a lower abundance leads to higher permeability of the membrane. In addition, a high fat diet increases the production of chylomicrons and causes their accumulation in the paracelullar space, also loosening the tight junctions (Boroni Moreira et al. 2012). Thus, a high fat diet leads to an increased permeability of the epithelium and allows the passage of toxins like lipopolysaccharide (LPS). This molecule is produced by Gram negative bacteria, transported to the blood stream by chylomicrons and thus seems to be a key molecule in the appearance of the typical low grade inflammation observed after high fat feeding.

Short-chain fatty acids, the end-products of microbial fermentation, are important energy sources for the host. Indeed, acetate will mainly be transported to the liver where it will be used as energy source but also as a precursor for other molecules production, including cholesterol; propionate will be used for gluconeogenesis (den Besten et al. 2013) and butyrate is the preferred energy source of enterocytes (Guilloteau et al. 2010b) and exerts anti-inflammatory properties by activating the

intestinal alkaline phosphatase, an intestinal enzyme responsible for the detoxification of LPS (Hinnebusch et al. 2003; Melo et al. 2016).

As microbiota is very important for barrier function, immune response and bodyweight gain, a strategy to avoid low-grade inflammation and obesity is to act on microbiota. For this, several solutions are envisaged that are mainly nutritional, using either probiotics (living beneficial bacteria, e.g. *Bifidobacterium*) or prebiotics (Mulders et al. 2018). Prebiotics are substrates that escape enzymatic digestion, are fermented by the microbiota and selectively promote the growth of beneficial bacteria within the microbiota at the expense of pathogens (Gibson et al. 2004). The establishment of a beneficial microbiota occurs early in life and there exists a transfer of bacteria from the sows to the progeny, as piglets are in contact with sows' faeces during the whole lactation period. Thus, reaching a beneficial microbiota for piglets with long lasting effects could be achieved by the use of prebiotics in sows' diet, with the hypothesis that these prebiotics could positively affects sows and thus piglets' microbiota (Schokker et al. 2014; Arnal et al. 2015). In this study, the emphasis was put on the use of dietary fibre in the form of resistant starch (RS) in the diet of sows in order to promote the establishment of a beneficial microbiota as early in life as possible for their progeny. Resistant starch can be defined as the proportion of starch able to escape enzymatic digestion due to its chemical and physical properties and can thus be fermented in the large intestine. It is considered as a prebiotic as it can selectively favour beneficial bacteria that exert fermentative and anti-inflammatory properties.

Thus, the research question of this study was to determine whether the inclusion of resistant starch in the diet of gestating and lactating sows could impact the ability of the piglets to cope with a high fat challenge, alleviating the symptoms of low-grade inflammation and/or obesity, by acting on the microbiota.

2. Materials and methods

2.1. Animals, diets and housing

Twenty-four Landrace sows were used during this animal experiment; they were inseminated with Piétrain semen, housed in groups on straw from 3 days after artificial insemination (AI) until one week before farrowing where they were housed in individual farrowing units equipped with wood shavings and a heat lamp for piglets. Sows and piglets were housed at the Walloon agricultural research centre in Gembloux (Belgium). All procedures led on the animals were approved by the ethical committee of the University of Liège (protocol n°1661). Sows were fed a standard diet during gestation until day 88. At day 88 of gestation, sows were divided in two groups, one group (n=12) receiving a diet containing 33% of standard maize starch, considered as digestible (DS) while the other group (n=12) received a diet rich in pea starch (Nastar, Cosucra, Belgium), considered as resistant starch (RS) until the end of lactation (28 days). The diets were adapted for nutritional requirements of sows

for the gestation and lactation periods (see composition in **Table 20**). At weaning (28 days of age), 44 female piglets (22/DS sows, 22/RS sows, 6 sows/treatment) were moved to the centre for animal production of Liège University in Gembloux. The temperature on the day of arrival was of 26°C, piglets were housed on grates, two littermates being housed in each pen.

Piglets were fed a standard weaning diet during 21 days, devoid of antibiotics, organic acids or NSP enzymes. At day 22 post weaning (PW), piglets were fed either a control diet (CON) containing 10% of maize starch (Roquette, Lestrem, France) or a high fat diet (HF) containing 10% of palm oil (Mosselman S.A., Ghlin, Belgium) to induce a high fat challenge. During this challenge, the feeds provided were a grower 1 diet (11-25 kgs) and followed by a grower 2 diet (25-50 kgs) to reach their nutritional requirements considering their bodyweight. The animal experiment ended 10 weeks post-weaning with slaughtering. Five piglets facing too severe weight loss after weaning had to be treated with antibiotics and were thus out of the experiment, while one pig died by sudden death.

2.2. Piglets' performances

Piglets' bodyweight was recorded weekly during the whole experiment. Two days before slaughtering, the backfat , muscle thicknesses and meat percentage were determined using a Piglog 105 lean meater (Carometec, Smørum, Denmark). The number of pigs affected by umbilical hernia was also recorded at the end of the experiment.

2.3. Blood sampling and analysis

Six weeks after the beginning of the high fat challenge, piglets were fasted for 12 hours and blood was sampled in 9-ml serum tubes (S-monovette, Sarstedt, Germany). After centrifugation at 2000g for 10 minutes, the supernatant (serum) was collected and stored at -20°C until further analysis. Serum triglycerides (TG), total cholesterol (TC) and high density lipoprotein (HDL) were analyzed with TRIGL, CHOL2 and HDLC3 packs on a Cobas 8000 instrument (Cobas, Roche, Switzerland). The low density lipoprotein (LDL) concentration was calculated following Friedewald *et al.* (1972) formula: LDL=TC-HDL-TG/5.

2.4. Sampling of intestinal tissue and content

At the end of the experiment (10 weeks post-weaning), the 38 remaining piglets were firstly profoundly anesthetized with a mix of Xylazine and zoletil 100 (4 mg of xylazine, 2 mg of zolazepam and 2 mg of tilamine/kg) and were then euthanized by bleeding. . The length of the intestinal tract was measured and the pH of the ileum and proximal and distal colon was measured using a pH-meter. Contents of caecum, proximal and terminal colon were collected in sterile ml tubes and stored at -80°C until further analyses. Mucus of the proximal colon was obtained after emptying the

colon with saline solution and scratching on a cold surface; mucosa was then snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Table 20. Ingredients and analyzed chemical composition of grower diets fed to pigs.

Ingredient	Grower 1 (11-25 kg)		Grower 2 (25-50 kg)	
	CON	HF	CON	HF
Barley	17.7	17.7	11.25	11.25
Maize	13.5	13.5	9.4	9.4
Wheat	13	13	27	27
Soybean meal 48%	12.1	12.1	14.2	14.2
Palm oil	-	10	-	10
Maize starch	10	-	10	-
Golden soy	9	9	0	0
Nutex 68 (Dumoulin Inc)	4.5	4.5	0	0
Bread flour	4.5	4.5	4.95	4.95
Biscuit flour	4.5	4.5	4.95	4.95
Wheat bran	3.6	3.6	2.34	2.34
Rapeseed meal	2.2	2.2	4.95	4.95
Sunflower meal	0	0	3.15	3.15
Chalk	1.5	1.5	1.36	1.36
Beet pulp	1.3	1.3	1.8	1.8
Fat	0	0	1.67	1.67
Maize gluten	0	0	1.188	1.188
Amino acids (Thr, Try, Met, Lys)	1.132	1.132	0.775	0.775
Minerals & Vitamins	0.603	0.603	0.369	0.369
Salt	0.4	0.4	0.333	0.333
Molasses	0	0	0.36	0.36
Analysed chemical composition				
DM (%)	88.8	89.7	89.94	89.41
CP	18.03	17.81	18.40	18.84
EE	5.05	15.94	4.77	14.88
ADF	5.15	5.55	5.29	5.37
NDF	12.69	13.83	13.65	14.65

2.5. Short-chain fatty acids (SCFA) determination

Piglets' caecal and colonic contents were diluted in ultrapure water to reach a 5-fold dilution before passage on HPLC for SCFA determination. Samples were centrifuged at 13,000g for 15 minutes, acidified and filtered on 0.22µm filters. The quantitation of SCFA was performed on isocratic HPLC equipped with a Waters system fitted with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) combined with a UV detector (210nm). Sulfuric acid (5mM) was used as mobile phase at a flow rate of 0.6ml/min. SCFA concentrations were quantified by integration of each peak with Empower 3 software (Waters, Milford, USA) after encoding a standard curve. The results are transformed to be expressed in $mmol.g^{-1}$, taking into account the sample dilution. Molar ratios were then calculated for every SCFA.

2.6. Microbiota composition and calprotectin concentration

DNA was extracted from proximal colon of 32 piglets (8/treatment) using QiagenQIAamp Stool Minikit (Qiagen, Hilden, Germany) following the manufacturer's recommendations with the addition of two bead beating steps (FastPrep-24, MP Biomedicals, Illkirsh, France) during 3 minutes by 30" intervals with 30" intermittent cooling, at a speed of 6M/s. Quality of DNA was checked on 1% agarose gel and the DNA concentration was assessed by a Nanodrop (Thermo Scientific NanoDrop 2000, USA). DNA was stored at -20°C until sequencing. Sequencing was performed by DNAVision (Gosselies, Belgium), using the Illumina MiSeq (2 × 300nt) and after amplifying, purifying and tagging the hypervariable regions V3-V4 (Forward primer: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGC AG-3′ and reverse primer: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATC TAATCC-3′) following the 16 S Metagenomic Sequencing Library Preparation protocol (Part # 15044223 Rev. B) from Illumina. Calprotectin concentration in the colon contents of the piglets was assessed using Porcine Calprotectin *ELISA* Kit (MyBioSource, San Diego, USA) following the manufacturer's recommendations. Absorbance was measured at 450nm.

2.7. Gene expression

RNA was extracted from proximal colon mucosa with ReliaPrep™ RNA Tissue Miniprep System kit (Promega, Madison, USA) using bead beating to disrupt the tissue. The concentration of RNA was assessed on a Nanodrop (Thermo Scientific Nanodrop 2000, USA) and all concentrations were aligned in order to use 1µg of RNA in the reverse transcription. The RNA integrity was verified on 1% agarose gel. RNA was then retro-transcribed into single-stranded cDNA with GoScript™ Reverse Transcription Mix (Promega, Madison, USA). Specific regions of cDNA coding for

house keeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 400nM) and β-actin (ACTB, 300nM), for tight junction proteins zonula occludens 1 (ZO1, 200nM) and occludin (OCLN, 200nM) and for cytokines and molecules related to inflammation interferon-γ (IFNγ, 400nM), Tumour Necrosis Factor α (TNFα, 200nM) and Interleukin 1β (IL1β, 400nM) were amplified with qPCR using the SYBR Premix Ex Taq II (TakaraBio) with 3-steps: 95°C for 5", 60°C for 30" and 72°C for 30". All primers efficiencies were optimized between 90 and 110% and their specificity was checked on the melting curve. GAPDH and ACTB were used as reference genes after confirming their stability and the $2^{-\Delta\Delta Ct}$ method was used. The list of primers is available in Table 21.

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2.8. Bioinformatics and statistical analyses

Raw sequences of 16S rRNA were assigned to each sample, quality checked and trimmed using Basespace default parameters (Illumina). Sequences were assigned to 97% ID OTUs by comparison to the Greengenes reference database 13.8 using the QIIME (Quantitative Insights Into Microbial Ecology) 1.9.0 software. Since samples contained variable number of sequences (90903±5396), diversity analyses were carried out on samples rarefied at the same sequencing depth (47788) to avoid bias in sequencing depth between samples. The Beta_diversity_through_plots.py script was used to assess differences in bacterial communities and functional composition between groups of samples. Beta diversity was visualized using un-weighed and weighed UniFrac distances with Principal Coordinate Analysis (PCoA). The compare_categories.py script, which applied the adonis method on the previously obtained dissimilarity matrices, was used to determine whether communities differed significantly between groups of samples. Multiple_rarefactions.py and alpha_diversity.py scripts were applied to compute alpha diversity metrics, which evaluated diversity within a sample and generated rarefaction curves. For microbiota composition, a non-parametric test was used as normality of the data was not achieved. For this, the proc NPA1WAY of SAS was used, either considering the maternal diet, the pig diet or both in a 4-treatments setup, non-parametrical tests not allowing the 2-ways analysis.

For the other parameters, all statistical analyses were performed with SAS 9.2 software (SAS Inc, USA) using the MIXED procedure of SAS, including the maternal diet and the pig treatment in a two-ways ANOVA. Normality of the data was tested with Shapiro-Wilk's test and variance equality with Levene's test.

Table 21. Primers used for the qPCR analysis.

Primer		Sequence (5'->3')	Reference	Accession number
ACTB	*F*	GGA-CTT-CGA-GCA-GGA-GAT-GG	Dozois et al. (1997)	XM_021086047
	R	GCA-CCG-TGT-TGG-CGT-AGA-GG		
GAPDH	*F*	CAT-CCA-TGA-CAA-CTT-CGG-CA	Chatelais et al. (2011)	NM_001206359.1
	R	GCA-TGG-ACT-GTG-GTC-ATG-AGT-C		
TNF-α	*F*	ACT-GCA-CTT-CGA-GGT-TAT-CGG	Meissonnier et al. (2008)	NM_214022.1
	R	GGC-GAC-GGG-CTT-ATC-TGA		
IFN-γ	*F*	TGG-TAG-CTC-TGG-GAA-ACT-GAA-TG	Royaee et al. (2004)	NM_213948
	R	GGC-TTT-GCG-CTG-GAT-CTG		
IL1β	*F*	ATG-CTG-AAG-GCT-CTC-CAC-CTC	Gourbeyre et al. (2015)	NM_214055
	R	TTG-TTG-CTA-TCA-TCT-CCT-TGC-AC		
ZO-1	*F*	TGA-GAG-CCA-ACC-ATG-TCT-TGA-A	Vigors et al. (2016)	XM_021098856
	R	CTC-AGA-CCC-GGC-TCT-CTG-TCT		
OCLN	*F*	CTA-CTC-GTC-CAA-CGG-GAA-AG	Chen et al. (2013)	NP_001157119.1
	R	ACG-CCT-CCA-AGT-TAC-CAC-TG		

3. Results

3.1. Performances

Table 22. Backfat thickness, muscle thickness, meat percentage and bodyweight of pigs after the high fate challenge. (n=9 for DS CON, DS HF, RS CON and n=11 for RS HF)

Treatment	BFT[1]	MT	Meat %	BW 10 weeks PW
DS CON	6.0	42.3	63.5	37.6
DS HF	7.1	44.0	62.7	35.4
RS CON	5.8	40.6	63.4	38.0
RS HF	8.5	42.5	61.5	36.2
Global SEM	0.3	1.0	0.2	0.9
P-values				
Maternal treatment	NS	NS	NS	NS
HF treatment	**<0.001**	NS	**<0.001**	NS
Interaction	NS	NS	NS	NS

[1]BFT: backfat thickness (mm), MT: muscle thickness (mm), BW: bodyweight (kg)

The HF challenge impacted backfat thickness and the meat ratio ($p<0.001$, Table 22), as challenged pigs had a higher BF thickness (5.9 ± 0.2mm for CON pigs *vs* 7.9 ± 0.4mm for HF pigs) and subsequent lower meat percentage (63.5 ± 0.3% for CON pigs *vs* 62.0 ± 0.3% for HF pigs). Muscle thickness and final bodyweight at slaughtering were not impacted by the maternal or high fat treatments. Neither the maternal nor the pig diets impacted the weekly bodyweight gain of the animals (data not shown).

3.2. Cholesterol

Table 23. Total cholesterol (mg/dl), triglycerides (mg/dl), high density lipoprotein (mg/dl), low density lipoprotein (mg/dl) and ratio in pigs' fasted plasma after 6 weeks of HF challenge (n=8/treatment).

Treatment	TC[1]	TG	HDL	LDL	ratio LDL/TC
DS CON	95.3^{ab}	40.7^{b}	46.3^{b}	40.8^{a}	42.6^{a}
DS HF	105.3^{a}	44.0^{b}	58.7^{a}	37.8^{ab}	35.9^{b}
RS CON	86.4^{b}	38.3^{b}	43.8^{b}	35.0^{ab}	40.4^{ab}
RS HF	101.7^{a}	54.0^{a}	59.6^{a}	31.3^{b}	30.1^{c}
Global SEM	2.66	1.91	1.90	1.55	1.27
P-values					
Maternal treatment	NS	NS	NS	**<0.05**	**<0.05**
HF treatment	**0.01**	**<0.01**	**<0.001**	NS	**<0.001**
Interaction	NS	0.07	NS	NS	NS

[1]TC: total cholesterol; TG: triglycerides; HDL: high density lipoprotein; LDL: low density lipoprotein

Within every treatment in the same column, values having a different superscript letter are significantly different ($p<0.05$).

Total cholesterol, triglycerides and HDL concentrations increased with HF challenge (Table 23), while the ratio between LDL and total cholesterol decreased. Moreover, LDL was decreased for piglets born from RS sows compared to DS sows, leading to a lower LDL/TC ratio for these pigs.

3.3. Microbiota and SCFA production

No differences in microbiota diversity or richness were observed between dietary treatments ($p<0.10$). The global microbiota composition was not affected by the maternal or pig treatment either, as observed by the PCoA in the Figure 18.

At the phylum level, this was translated by no impact of maternal diet or HF challenge on the relative abundance of the different phyla, as presented in Figure 19. Only the minor phylum *Planctomycetes* was affected by the pig treatment (0.019 ± 0.005 for CON; 0.004 ± 0.001 for HF pigs). The ratio between *Firmicutes* and *Bacteroidetes* was not impacted ($P>0.05$) by the treatments either (1.71 ± 0.18 for DS CON; 1.39 ± 0.09 for DS HF; 1.58 ± 0.14 for RS CON; 1.64 ± 0.14 for RS HF).

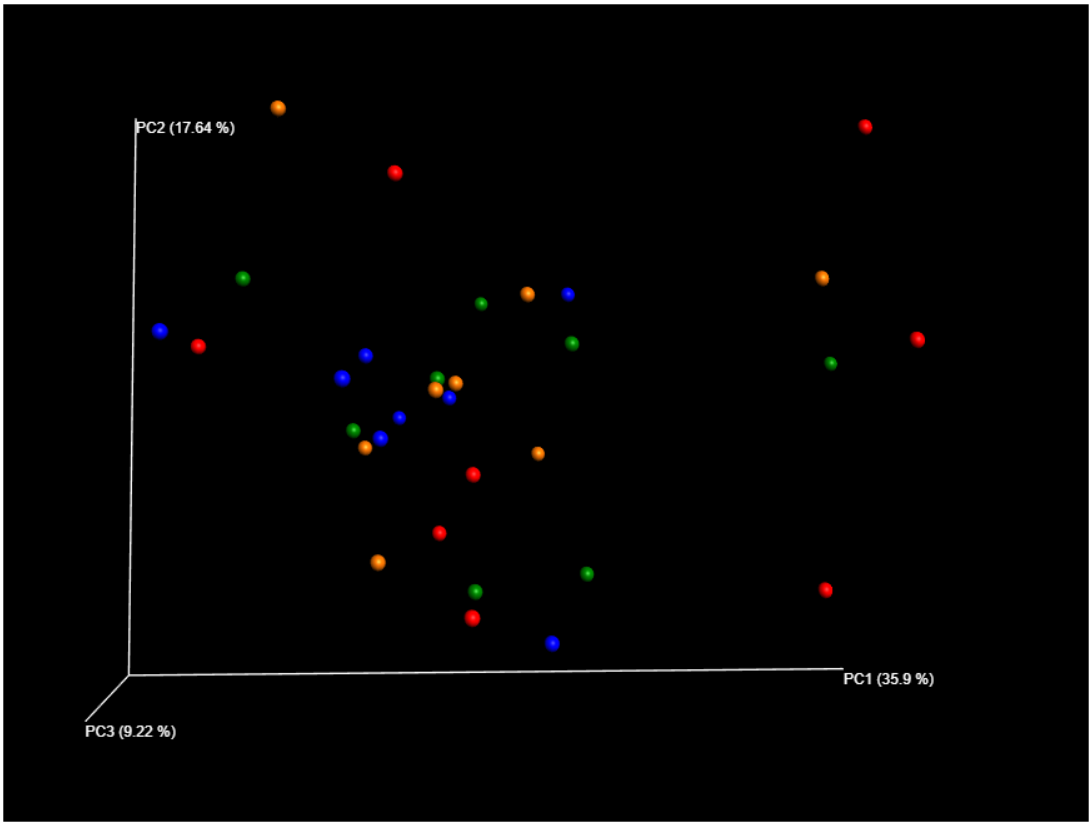

Figure 18. Principal component analysis of the microbial composition of the colonic contents. Red dots are DS CON pigs, blue dots are DS HF pigs, orange dots are RS CON pigs, green dots are RS HF pigs (n=8/treatment).

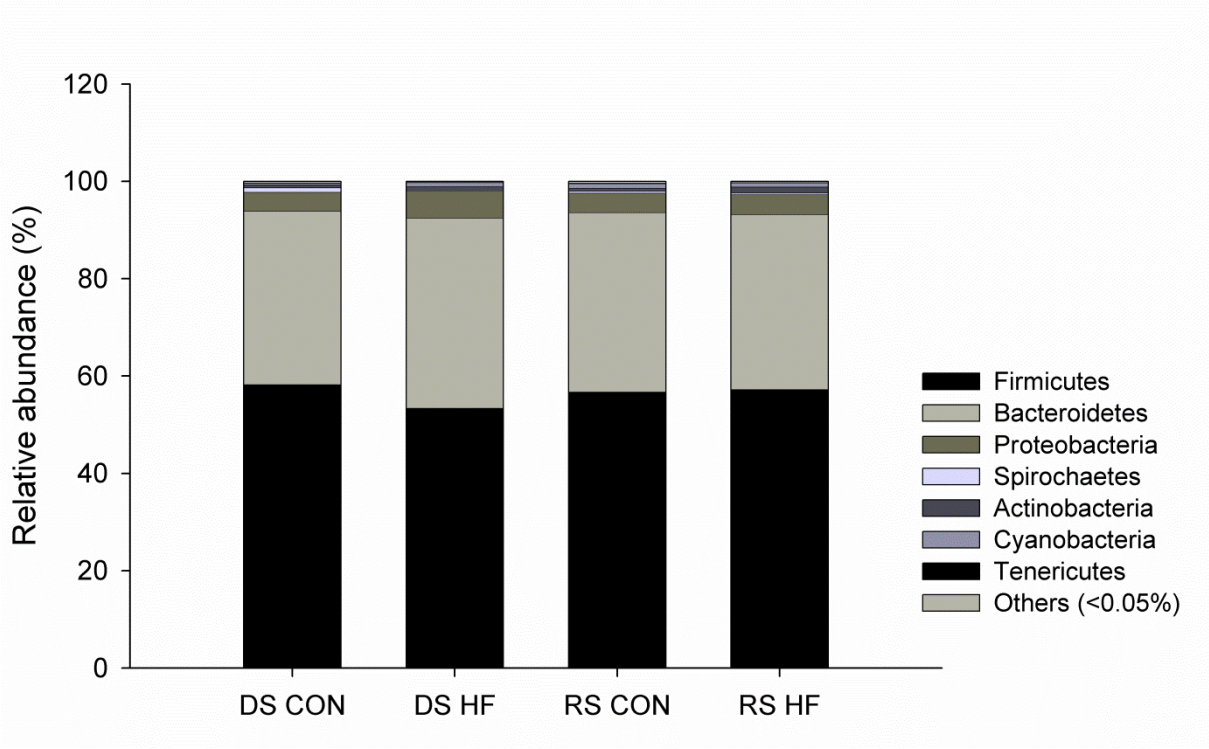

Figure 19. Proportion of the main abundant phyla in the different pigs groups (n=8/treatment).

Maternal treatment impacted the total production of SCFA in the caecum and proximal colon of the pigs (Table 24), pigs born from RS sows having a higher SCFA production than pigs born from DS sows. An interaction between maternal and pig treatment ($p=0.04$) was observed in the caecum as within the HF treatment, pigs born from RS sows had a significantly ($p<0.01$) higher total SCFA production than pigs born from DS sows. In the caecum of pigs, acetate and propionate productions were impacted by the high fat challenge, as propionate production was increased in challenged pigs at the expense of acetate. In the distal colon, acetate production was however differentially affected (interaction $p=0.02$): within the DS pigs, challenged pigs had a significantly lower acetate production ($p=0.048$) than CON pigs; within the HF treatment, RS_HF pigs had a significantly higher acetate production than DS_HF pigs ($p=0.03$). Butyrate production was not affected by either of the treatments within all intestinal segments.

Table 24. Total SCFA production and molar ratios of acetate, propionate and butyrate in the large intestinal contents of the pigs born from DS or RS sows and fed CON or HF diets.

	Maternal treatment	Pig treatment	Sum	% acetate	% propionate	% butyrate
Caecum	DS	CON (n=8)	9.24^{ab}	62.49	27.03	10.08
		HF (n=9)	7.62^{b}	59.18	29.50	9.73
	RS	CON (n=9)	9.29^{a}	59.52	28.24	10.19
		HF (n=12)	10.09^{a}	56.00	29.89	9.19
		Global SEM	0.31	0.85	0.44	0.27
	P-values	P mother	**0.03**	0.06	NS	NS
		P pig	NS	**0.04**	**0.02**	NS
		P interaction	**0.04**	NS	NS	NS
Proximal colon	DS	CON (n=8)	8.89	60.40	28.02	10.54
		HF (n=9)	8.76	58.30	28.55	10.26
	RS	CON (n=9)	9.58	59.30	28.69	10.53
		HF (n=12)	10.29	56.20	29.31	9.50
		Global SEM	0.23	0.76	0.46	0.27
	P-values	P mother	**0.01**	NS	NS	NS
		P pig	NS	0.09	NS	NS
		P interaction	NS	NS	NS	NS
Distal colon	DS	CON (n=8)	7.93	55.01^{a}	22.64	14.23
		HF (n=9)	8.89	51.03^{b}	23.23	16.24
	RS	CON (n=9)	9.35	52.51^{ab}	21.97	16.36
		HF (n=12)	8.61	54.91^{a}	22.95	15.11
		Global SEM	0.22	0.68	0.36	0.44
	P-values	P mother	NS	NS	NS	NS
		P pig	NS	NS	NS	NS
		P interaction	0.06	**0.02**	NS	0.07

3.4. Intestinal inflammation and permeability of the colon

The maternal treatment significantly impacted the gene expression in the colon of challenged pigs (Figure 20). Indeed, TNF-α, ZO1 and OCLN were more expressed ($p<0.05$) in the colon of pigs born from RS sows, while IFNγ was down-expressed in the colon of these pigs ($p<0.05$). IL1β was not impacted by the maternal diet. None of these genes expression was impacted by the high fat diet. Calprotectin concentration tended ($p=0.08$) to be lowered in the faeces of pigs born from RS sows (87.8 ± 2.6 ng/ml for the DS pigs vs 82.1±1.7 ng/ml for the RS pigs).

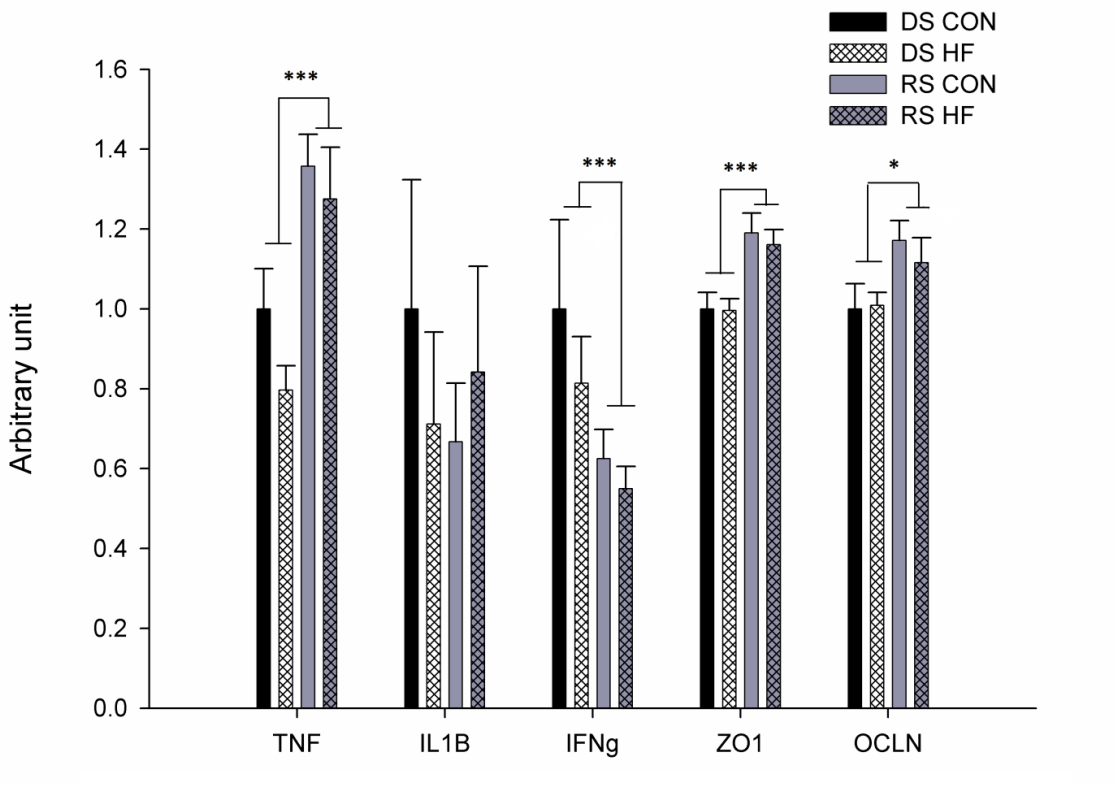

Figure 20. Gene expression of inflammatory cytokines and tight junction proteins in the proximal colon of pigs (n=8/treatment). P<0.05 is represented by * and P<0.001 is represented by ***.

4. Discussion

The research question of this study was to determine whether maternal resistant starch supplementation could alleviate the adverse consequences of a high fat challenge to their progeny later in life. Indeed, the health and metabolism of piglets could be impacted by the sows' diet and early environment as proposed by the theory of developmental origins of health and disease (Gluckman & Hanson 2006). This question relies on the hypothesis that resistant starch can promote beneficial microbiota in the sow gut that will colonize the gut of their offspring early in life, with long lasting effects on gut health, as bacteria are involved in SCFA production (den Besten et al. 2013), immune modulation (Everaert et al. 2017) and barrier function (Boroni Moreira et al. 2012). Alternatively, the maternal treatment could program the metabolism of the progeny.

The primary question was whether the applied HF challenge was sufficient to induce early physiological effects related to fatness/obesity (namely increased backfat thickness and cholesterol) and low grade inflammation. Then, the effects of the maternal diet on these symptoms were investigated.

The HF challenge seemed to be promising for inducing dyslipidaemia, as total cholesterol, triglycerides and HDL production were increased in HF pigs' plasma. The ratio between LDL and TC was impacted by the HF diet and was surprisingly lower for HF pigs, which could be attributed to hepatic regulation following a chronic high fat exposure (Panasevich et al. 2018). Moreover, the backfat deposition was increased after 7 weeks of HF challenge. Dyslipidaemia and increased body fat are indicative of development of obesity (Torres-Rovira et al. 2012) but possibly the experiment did not last long enough to induce inflammation of the gut. Alternatively, liver samples could be more indicative of low grade inflammation and could give more insights in establishing whether the high fat challenge was severe enough. RNA-seq results on liver samples are still under analysis and could thus give us better insights on the inflammatory status and the metabolic programming of the animals.

In addition, HF diet did not affect microbiota composition, nor the ratio *Firmicutes:Bacteroidetes*. This ratio is however being questioned (Panasevich et al., 2008) for being a suitable indicator for obesity, as different studies on pigs and humans did not observe this shift in obese subjects (Heinritz et al. 2016; Lopez-Contreras et al. 2018). The impact of feeding HF diets on the microbiota differs widely depending on the fat source, percentage of inclusion in the diet and animal species used. For example, Feng et al. (2015) only observed a minor difference in microbiota composition when feeding growing pigs 5% soybean oil compared to a basal diet, while Hamilton et al. (2015) observed an effect on the abundance of several microbial genera in the caecum of rats fed 45% of fat. Taking the results of the microbiota together, the HF challenge did not induce profound changes in microbial structure. In addition, the maternal diet did not induce changes in the microbial composition of the progeny either. Maternal transfer is thought to occur mainly during the lactation period, with sows' faecal microbiota being in contact with

piglets and milk nutrients playing a role. Previously, we showed that the inclusion of resistant starch in gestation and lactation diets induced changes in faecal microbiota of sows fed RS during gestation but this differential microbiota was not maintained throughout lactation and did not affect the microbiota of the offspring at 26 days of age (Leblois et al. 2018). In line with this, no maternal effect appeared either on week 10 post-weaning, a time point with a more stable microbiota than at weaning (Bian et al. 2016); these results are in line with Arnal et al. (2015) who fed antibiotics to gestating and lactating sows without an impact on offspring's colonic microbiota on the short (14, 21, 28 and 42 days of age) and long (169 days of age) term.

Even though the microbiota was not affected by the maternal and HF treatments, SCFA production was impacted by both. High fat challenge impacted individual molar ratios of SCFA in the caecum of pigs: acetate proportion was decreased with the HF diet, together with a higher propionate production. These individual SCFA have been related to cholesterol production. Indeed, a direct acetate supplementation in the diet was shown in humans to reduce hypercholesterolemia (Kondo et al. 2009). On the opposite, propionate has been shown to be negatively correlated with cholesterol production (den Besten et al. 2013). Thus, the lower acetate production could be related to higher cholesterol while higher propionate should be related to lower cholesterol production. Even though these effects seem to be antagonistic, we observed in this study an increase of total and HDL cholesterol and triglycerides blood concentrations. In addition, acetate has also been shown to be related to a lower BW and fat deposition in human subjects (Kondo et al. 2009). In this study, pigs fed the HF diet and having the lower acetate production had the greatest backfat deposition.

On the other hand, maternal diet only impacted total SCFA production, as pigs born from RS sows had a higher total SCFA production than pigs born from DS sows, which is in line with Le Bourgot et al. (2014) who fed sows short chain fructo-oligosaccharides. This effect can be considered as beneficial as increased SCFA production is related to lower risk of metabolic diseases and cancer (Heinritz et al. 2016). Even though the maternal diet did not impact the microbiota composition nor the molar ratios of individual SCFA, the decreased LDL concentration and LDL/TC ratio for pigs born from RS sows suggest a metabolic programming of the piglets, which might be beneficial for their health as LDL is considered to be the "bad" cholesterol promoting atherosclerosis (Puccinelli et al. 2015).

Moreover, parameters related to inflammation and permeability of the gut mucosa were impacted by the maternal diet. Indeed, maternal RS might have decreased intestinal permeability as the expression of both OCLN and ZO1, part of the tight junction complex, increased. Impact of the maternal diet on transcellular permeability but not on paracellular was reported by Arnal et al. (2015) on the long term (169 days of age of the piglets) but the HF diet did not induce changes either. While HF feeding did not impact the inflammatory status of the colon, maternal RS diet increased TNF-α expression but lowered IFNγ expression. Other studies feeding sows prebiotics pointed out a more developed immune system for the piglets born from the

supplemented sows (Le Bourgot et al. 2014), increased ileal expression of IFNγ and TNF-α (Heim et al. 2015b) or no impact of the maternal diet (Leonard et al. 2012). All these studies were led on nearly weaned piglets, with digestive tract and gut immune system not being fully developed, rendering the comparison difficult. RNA sequencing of liver samples could give use better insights in the role of maternal diet on the development of low grade inflammation for HF treated pigs and on the metabolic programming in general.

To summarize, sows' supplementation and later high fat diet did not impact significantly the microbiota of the pigs. Interestingly, maternal diet increased total SCFA production, which is health-promoting and at the same time induced a decrease of LDL and LDL/TC ratio, together with decreased colonic permeability, suggesting a maternal nutritional programming of the progeny. RNA sequencing results on liver samples will bring further information concerning the development of other symptoms related to metabolic troubles and the impact of maternal diet on the metabolism of their progeny.

5. Acknowledgments

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8

General discussion and perspectives

Overview

During this PhD, the focus was put on the early programming of piglets' intestinal microbiota and health using the mother as an indirect strategy affecting bacteria and nutrients transmission. Indeed, the hypothesis was that introducing dietary fermentable fibres in sows' diet during pregnancy and lactation would alter their microbiota and milk composition that would in turn allow the establishment of a beneficial gut microbiota as early in life as possible, as piglets' microbiota is acquired from their mothers' (contact with maternal faeces) and is also affected by milk composition. Furthermore, an altered microbiota and/or milk composition could allow piglets to cope the weaning stress as these parameters influence gut architecture and immune system.

Moreover, an altered microbiota could also have the ability to help pigs cope with a dietary fat challenge later in life which was tested in the second animal experiment. This experiment aimed at inducing inflammation and obesity in pigs, using this animal as a model for Human.

The main results of the short-term experiments for two animal trials are summarized in Figure 21 and Table 25 and will be discussed below.

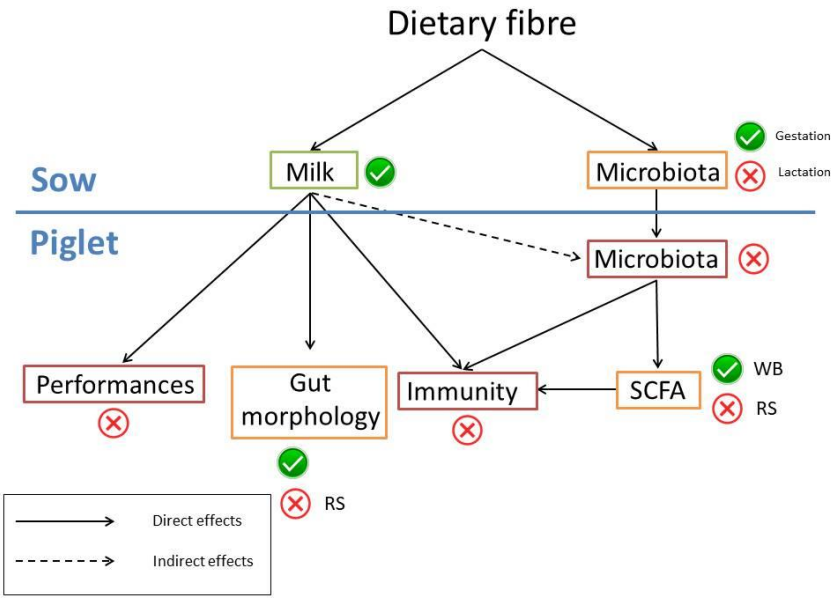

Figure 21. Main results of the two animal experiments led during this PhD thesis. Green V signs indicate a change observed for the studied parameter while the red X sign indicate no change.

Table 25. Main results of the PhD thesis with the two animal experiments using either wheat bran (WB) or resistant starch (RS) until weaning

Parameters		WB	RS
% fibre inclusion in the diet	- Gestation	24%	33%
	- Lactation	14%	33%
Time of supplementation		Day 43 after AI	Day 88 after AI
Microbiota of sows	- Gestation	PCoA clustering	PCoA clustering
		No phylum difference	Phyla differences: ratio *Firmi/Bactero* increased for RS
		13 genera significantly different	14 genera significantly different (*Bifidobacterium*)
	- Lactation	no PCoA clustering	no PCoA clustering
		No phylum difference	No phylum difference
		2 genera significantly different	6 genera significantly different
Milk composition	- Fat	Only parity effect	Trend for parity effect only
	- Lactose	Globally increased for WB	Increased RS sows first two weeks, decreased last week
	- Protein	Only parity effect	Lower for RS
	-Ig	/*	Trend for lower IgA in RS
Microbiota of piglets		No phylum difference	No phylum difference
		Trend for 4 genera	No genera difference
Gut morphology	- Villus height	Higher duodenum	/
	- Crypt depth	/	/
	- Ratio V:C	Higher duodenum & jejunum	/
Gene expression	- Inflammation	/	/
	- Tight junctions	/	Higher ZO-1 expression in RS piglets' ileum

*/ : no impact with the sows' diet supplementation

Choice of ingredients and percentage of inclusion in the diet

Two ingredients were used during this PhD, namely wheat bran and pea starch. These ingredients were selected on their ability to be fermented in the large intestine of the pigs and their aptitude to favour the growth of beneficial bacteria. The choice of WB and RS was based on literature (see point 4 from the introduction). For RS, different sources are available. This choice was made after *in vitro* fermentation in which pea starch showed the best ability to be largely and rapidly fermented, together with a high proportion of butyrate produced during fermentation compared to other RS sources (see chapter 5). The incorporation rate differed for both animal experiments. The purpose was to include as much fermentable ingredient as possible. For WB, as it is already used at a rate of around 15% in sows' diet during gestation, we tested 24% as diets containing 24% of dietary fibre had already been reported without impairing production parameters (Loisel et al. 2013). During lactation, as the sow needs more energy, we were limited to incorporation of 14% of WB in the diet, as wheat bran also contains other non-energetic compounds, such as lignin (Kamal-Eldin et al. 2009). In contrast with the complicated WB diet formulation, RS diet was much easier to formulate as a complete replacement of DS by RS was done. As RS does not contain lignin or any other non-energetic ingredients, the high incorporation rate (33%) during gestation could be carried on during lactation. This high incorporation rate was based on literature as higher rates had already been reported for sows or pigs (70%, 34% or 65 % of RS in the diet) with associated microbial changes (Regmi et al. 2011; Haenen et al. 2013; Yan et al. 2017, respectively)

Microbiota of the sow and offspring

Two animal experiments were conducted, with wheat bran and resistant starch as feed supplement in sows' diet. A first conclusion that can be drawn from the two animal experiments performed is that both WB and RS have the ability to impact sows' microbiota during gestation, this effect being stronger for RS than WB, as even the phyla abundance was impacted by the RS diet. Indeed, RS supplementation increased the proportion of *Firmicutes* to *Bacteroidetes*, which is an indicator for higher energy harvest from the diet (Maga et al. 2012), a beneficial effect for animal production. However, WB supplementation did not lead to the expected increase of butyrate-producing genera, namely *Clostridium*, *Anaerostipes*, *Blautia*, *Butyrivibrio*, *Coprococcus*, *Dorea*, *Lachnospira*, *Pseudobutyrivibrio*, *Roseburia*, *Faecalibacterium*, *Oscillospira*, *Ruminococcus*, *Megasphaera* and *Butyricimonas* (Louis & Flint 2009; Levine et al. 2013; Bian et al. 2016) or the relative abundance of health promoting bacteria (*Lactobacillus*, *Bifidobacterium*) even though *Lactobacillus* relative abundance in WB sows was higher than CON sows, without reaching significance, probably due to the high variability between animals. On the other hand, RS increased the abundance of *Bifidobacterium* and of the fermentative

family *Ruminococcaceae* during gestation. Thus, RS may be a more adequate candidate to be incorporated in the diet of animals to promote a healthy microbiota.

The **stronger impact of RS** on sows' microbiota during gestation could be the result of the higher incorporation rate in the diet (33% for RS *vs* 24% for WB) and a higher fermentability of the substrate. Indeed, WB is mainly composed of insoluble fibres; this type of fibre needs a longer transit time and the extent of degradation will depend upon the degree of lignification (Govers et al. 1999; Bach Knudsen & Canibe 2000) while RS is more rapidly available for degradation by bacterial fermentation (Govers et al. 1999). The main sites of fermentation of the two substrates also differ, as WB is mainly fermented in the colon and RS in the caecum (Govers et al. 1999; Haenen et al. 2013; Nielsen et al. 2014). Moreover, the amount of ADF between Con and WB diets did not differ, even though the formulation was supposed to induce differences in non-starch polysaccharide content. ADF and NDF measurements in the case of RS are not of interest, as they do not include the amount of RS available for fermentation. An interesting perspective would be the introduction of both WB and RS substrates in the diet of the animals, as WB introduction has been shown in growing pigs to shift RS fermentation from the caecum to distal colon, increasing butyrate production (Govers et al. 1999).

The two animal experiments were led on different sows, with different age and parity. It can actually be observed that for the CON sows of each experiment, the relative abundance of the phyla were different from one to another study despite the same trend for an increase and/or decrease of particular phyla between gestation and lactation in every experiment. For example, *Firmicutes* proportion was higher in the first animal experiment than in the second while *Bacteroidetes*, *Spirochaetes* and *Proteobacteria* were lower. Even though the control diet differed between experiments, this highlights the inter-studies variability.

Two supplementation durations were studied, as WB feeding began 43 days after AI and RS diet 88 days after AI. What is relevant from these two studies is that feeding sows the fibre diet one month before farrowing seemed sufficient to induce profound microbial (Phyla) changes. However, the time for adaptation should not be too short as Sappok et al. (2015) highlighted the need of more than 19 days for sows' microbiota adaptation and stabilization.

Even though microbiota differentiation between groups was observed for both ingredients during **gestation**, the clustering was not observed anymore during **lactation**. Using 3% inulin, Paßlack et al. (2015) observed a time effect (gestation *vs* lactation) for specific bacterial genera and had the same conclusion for two genera (*Enterobacteria, Enterococci*) for which a diet effect was observed during gestation but not anymore after farrowing. From the second animal experiment, it is clear that the global microbiota composition is different during gestation and lactation. In agreement with Tan et al. (2016), an increase in *Firmicutes* and a decrease in *Proteobacteria* relative abundances from gestation to lactation has been observed for both experiments (Tan et al. 2016). A differential microbiota composition has also been observed between early and late pregnancy in humans (Koren et al. 2012) and

sows (Larivière-Gauthier et al. 2017) as well as in ruminal bacterial communities of cows pre- and post-partum (Pitta et al. 2014). Mechanisms responsible for the microbial shifts are not well known, but it seems that the diet change happening for lactation may be responsible together with metabolic changes. It is noteworthy that stresses occurring at farrowing and during lactation (milking, piglets' handling) may also be partly responsible for the microbial shifts observed during the lactation period.

In addition, during the first animal experiment, wheat bran was replaced in the CON diet by other fermentable components such as cocoa pods, which might have contributed to the microbial differences between treatments observed during gestation, causing interferences in the study. In the future, testing the two types of diets *in vitro* before an animal experiment would be interesting to get insights in the fermenting ability of both supplemented and non-supplemented diets. Moreover, an ideal experimental setup would include the screening of faecal microbiota of sows before the diet change in order to select sows with the lowest inter-individual variations, but this would be costly and time consuming. In addition, the reliability would not be maximal as microbiota disruptions might be expected because of stress.

As no difference in microbiota composition remained between CON and supplemented sows during lactation, it might explain that microbiota of the piglets at weaning age (26 days old) did not differ to a large extend (WB) or not at all (RS) between piglets born from control or fibre sows. For future research, it would be interesting in the first place to describe the microbiota of sows around farrowing: a few hours before, during farrowing and a few hours after, to get a better picture of when this microbial shift happens. Then, it would be interesting to analyse the microbiota of the piglets within the first days of life, as their microbiota is closer to their mother's within the first days than after several weeks (Bian et al. 2016).

As microbiota of piglets at weaning barely (WB) or did not differ (RS) between maternal treatments, it is not surprising that the impact on the **SCFA** was limited. Indeed, for the WB experiment, only valerate was impacted in all intestinal compartments, as a lower concentration was observed for piglets born from WB sows, which can be considered beneficial as valerate is an end-product of protein fermentation that also produces toxic compounds (Yao et al. 2016). However, butyrate production was lowered in the caecum of WB piglets, which was not desired. RS supplementation on the other hand did not lead to any SCFA changes at weaning. However, on the long term (11 weeks after weaning), the maternal diet increased significantly the total production of SCFA in the caecum and proximal colon of pigs born from RS sows; this might be beneficial as a higher production of SCFA has been related to a lower risk of metabolic diseases and cancer (Heinritz et al. 2016).

Microbiota results thus suggest that WB and RS are good candidates to be incorporated in pigs' diet, but their effect on the progeny is rather limited. Therefore, using these ingredients in the diet of pre-weaned or newly weaned pigs may be a more promising approach to induce early colonization of beneficial bacteria within the intestinal microbiota and decrease the occurrence of PWD and the related antibiotics use. Particular attention should however be given to the palatability and digestibility of diets containing dietary fibres. As weaning is already associated with lower feed intake, the diets should be palatable enough to be ingested by the pigs.

Another hypothesis tested was that a **transfer of microbiota could already occur during gestation** and that piglets' GIT may not be completely sterile at birth as commonly considered. This hypothesis is based on the work of Jiménez et al. (2005, 2008) and Satokari et al. (2009) who found out bacteria in amniotic fluid, umbilical cord and placenta of women and in the meconium of new-borns. The analysis of umbilical cord blood in our first animal experiment revealed the presence of bacteria (including intestinal bacteria). However, in the colonic content of neonates we could not detect any DNA; this might be the result of the actual absence of bacteria within the GIT of the neonate or of a low sensitivity of the DNA extraction kit. Finding bacteria in all these neonatal/foetal tissues is new and also under contest (Willyard 2018), as some authors (Hornef & Penders 2017) highlight the need for controls all over the sampling and DNA extraction process as contamination of the material is very easy. However, as Jiménez et al. (2005) inoculated pregnant mice with labelled *E. faecium* strain that they found back in the amniotic fluid and neonate's meconium, it is reasonable to think that some bacteria may be transmitted to the offspring already before birth, even though the term "microbiota" may be overdone. Other researchers have also found bacteria in the placenta of woman after C-section (Satokari et al. 2009; Aagaard et al. 2014), supporting further this maternal pre-birth transfer. One mechanism underlying this transfer in human has been summarized by Thum et al. (2012) and relies on the uptake of bacteria from the intestinal lumen by Peyer's patches dendritic cells in the maternal gut, allowing a spreading in the whole body, including placenta, by the blood stream. However, the epitheliochorial placentation of the pigs may not allow the transfer of bacteria from the maternal to the foetal blood and may reinforce the contamination hypothesis; to our knowledge, no other study using animals harbouring epitheliochorial placentation could detect bacteria in foetal membrane, even though bacteria were found in the endometrium of healthy cows (Karstrup et al. 2017) and that viral and bacterial contaminations were reported for sows (see chapter 1, 2.1).

Thus, even though maternal transfer of microbiota during gestation is still not widely spread and accepted, it is important to continue research in this direction, using more precautions concerning air and material contamination and using also quantitative methods for bacterial concentration determination rather than relative sequencing (Hornef & Penders 2017; Willyard 2018).

Milk composition

Milk composition is very important for piglets' growth (milk yield and nutrients), for beneficial bacterial establishment (presence of fermentable compounds s.a. oligosaccharides) and for the immune status of the piglets (acquisition of passive immunity by ingestion of maternal immunoglobulins). Therefore, acting on sows' milk composition is crucial for the good development and health of the progeny. Several studies got interest in the modulation of milk nutrients and Igs composition by feeding sows different feed ingredients (dietary fibres, mannan, fat source – Loisel et al. (2013); Graugnard et al. (2014); Krogh et al. (2017), respectively). In the present studies, both WB and RS were able to impact nutrients composition of the milk, while effects on the Igs contents of colostrum were not significant. Fat percentage was neither affected by WB nor by RS supplementation; the protein percentage decreased for RS sows compared to DS sows while no impact was observed for WB supplementation. This lower protein percentage was translated by a trend for lower IgA concentration in colostrum. IgA is mostly locally produced in the mammary gland (secretory Iga, sIgA) and has been shown to reduce the concentration of pathogens in the faeces of piglets (Salmon et al. 2009). Thus, a lower IgA concentration in sows' colostrum is not desirable as piglets rely on passive immunity provided by their mother. However, as the actual analysed crude protein content of the lactation RS diet was slightly lower than DS, it cannot be excluded that the lower milk protein concentration is due to the lower CP content of the diet rather than to the incorporation of RS.

Lactose concentration was significantly impacted for both feed additives even though the numerical difference is rather small; WB increased globally lactose concentration while RS increased lactose in colostrum and milk sampled one week after farrowing while the opposite effect was observed during the last week of lactation. It is known that lactose is the less variable component in sows' milk as it is responsible for drawing water into milk (Hurley 2015; Theil et al. 2012) but it is also the less variable component between animals (Hurley 2015), probably explaining the significance of results for both animal experiments. Lactose is one of the only milk components that is not affected by parity of the sows (Hurley 2015) and this was observed for both animal experiments. Lactose synthesis in the mammary gland is independent on blood glucose concentration but will vary depending on the amount of GLUT1 transporters; these transporters abundance are in turn influenced by the development of epithelial mammary cells (Theil et al. 2012). Thus, an explanation for a higher lactose concentration in the milk during the first weeks after farrowing for WB and RS sows could reside in a better development of epithelial cells from the mammary glands, allowing an increase of GLUT1 transporters and thereby an increased concentration of lactose in milk. In addition to fat, lactose constitutes an important energy source for piglets within the first weeks and could then be related to piglets' growth. However, even though a globally increased lactose concentration was observed for high fibre sows, their piglets' weekly performances were not affected.

As a **parity effect** was observed for milk protein, fat and Igs contents for both experiments, attention should be paid in the future on performing animal experiments with sows from the same parity in order to decrease the variability between milk components to omit the different feed allowance and different BW.

The role of pro-inflammatory **cytokines** in milk is not totally understood; in the WB experiment, although results were not presented in paper 2, we quantified TNF-α concentration in colostrum and milk and found a high variability between animals, together with high number of undetectable values. TNF-α may not be a good indicator to determine health or sickness of the sow and piglet, as its role is unclear and variability is huge, as already shown in humans (Hawkes et al. 1999) and sows (Nguyen et al. 2007). Indeed, no relationship could be found in humans between the production of pro-inflammatory cytokines in milk and any illness of the individuals (Hawkes et al., 1999). Moreover, as TNF-α is locally produced in the mammary gland (Nguyen et al., 2007), it might reflect a local infection of the mammary gland or these maternal cytokines might play an activating role on the immature immune system of the new-borns, as proposed by Hawkes et al. (1999). In addition, Zabielski et al. (2008) highlighted that TNF-α also acts as a signal for epithelial cells apoptosis after birth, which is undesirable for the piglet, except if it contributes to gut closure by replacement of foetal-type to adult-type enterocytes.

It is important to note that all conclusions concerning milk composition should be taken carefully as the **milk yield** was not measured during these experiments. However, colostrum yield has been shown to be positively related to lactose concentration; thus it can be postulated that an increased milk yield is possible for fibre diets. Yet, measuring milk yield in the future may be interesting. This is possible by the calculation of the milk intake of the piglets with an equation developed by Devillers et al. (2004) widely used in studies (Foisnet et al. 2010; Loisel et al. 2013). Another perspective would be to determine the concentration of oligosaccharides in the colostrum, as they can be considered as prebiotics and are present in 29 forms in the sows' milk (Tao et al. 2010). Trying to develop equations to predict immunoglobulins in the colostrum of sows is also planned as it was already done for cow's colostrum (Elsohaby et al. 2018); the samples to increase the data set are still analysed by other teams. Moreover, even though sterile collection of milk samples was not possible during these experiments, it would be interesting in the future to achieve this in order to characterize the bacteria present in colostrum and milk, as it has been shown that a high amount of viable bacteria reach the mammary gland and are then major colonizers of the human and pig neonate gut (Mackie et al. 1999; Chen et al. 2018).

Gut morphology

Gut morphology is important for piglets' health as it is related to nutrients absorption and renewal of the intestinal epithelium. Thus, acting on gut morphology early in life can be beneficial; in particular, increasing villus height before the weaning transition may be a good strategy to avoid the alterations in villus/crypt ratio that appear at weaning. Firstly, milk trophic factors, including IGF-1 and lactoferrin can impact villus height in the progeny, as has been shown in humans (Li et al. 2017). Then, nutrients may also play a role on the gut morphology, as dietary fat has been shown to increase villus height and then absorptive capacity of the gut (Feng et al. 2015). An increased villus/crypt ratio can be associated with higher expression of nutrients transporters like SGLT1 (Heim et al. 2015a), therefore increasing the ability of the gut to digest feed. Then, microbiota present in the ileum, fermenting dietary undigested compounds, could also be related to gut morphology, and microbial exposure has been shown in germ-free mice to be related to shortening and widening of the villi that are part of increased mucus integrity (Bäckhed 2011). In the experiments led, only maternal WB had the ability to induce changes in piglets' gut morphology, as duodenal villi were higher while the ratio between villi and crypts was higher in the duodenum and jejunum of the piglets. It might be interesting to see whether this higher ratio could partially prevent the villi atrophy happening after weaning.

In the future, it may thus be more appropriate to measure the gut morphology after weaning in order to determine whether maternal diet could impact the severe villi shortening that commonly appears at weaning, even though the morphology before weaning can give us clues concerning the future ability of piglets to cope with the weaning transition. Measuring villus width, mucosa thickness or the number of goblet cells could also increase our knowledge concerning the ability of the epithelium to act as a barrier against pathogens and about the total absorptive surface of the gut.

Inflammatory status and mucosal integrity

In addition to gut morphology, tight junctions are indicators of a good barrier function of the mucosa, impairing the passage of antigens through the paracellular pathway. Any dysfunction of the barrier can lead to inflammation. Thus, increasing the expression of proteins like occludin and ZO-1 is important to reinforce the gut barrier and thus for piglets' health. Considering the two animal experiments led, only maternal RS diet increased the gene expression of these proteins in the ileum of pre-weaned piglets. This may indicate a higher integrity and lower permeability of the ileal mucosa and lower risk of inflammation, but this difference did not lead to differential diarrhoea scores. After weaning and the high fat challenge, maternal RS also increased the gene expression of both proteins in the colonic mucosa, without an impact of the high fat diet, which is in agreement with Arnal et al. (2014). For the future, using *in vivo* permeability measurements or Ussing Chambers would be more

informative; in particular, Ussing chambers allow the determination of permeability using low (FITC-dextran, FD4, 4kD) and high (horseradish peroxidase, HRP, 40kD) molecular weight molecules for trans- and paracellular permeability determination, respectively (Arnal et al. 2014) with an electrical flux. This *ex vivo* technique is widely used to assess intestinal permeability for pigs and rodents (Mangell et al. 2002; Wallon et al. 2005; Brun et al. 2007; Lallès et al. 2009).

To determine the ability of the piglets to cope with a bacterial infection, the intestinal tissues of the animals were cultured *ex vivo* with LPS toxin extracted from ETEC O111:B4 strain and incubated for 2 hours at 39°C, 5% CO_2. Our results showed that the LPS challenge did not increase the expression of TNF-α or TLR4 compared to the control explants, suggesting that LPS did not induce the expected inflammation. Other authors using LPS extracted from the same *E. coli* strain at the same concentration (10µg/ml) had contrasting results, showing an increased TNF-α expression (Mukhopadhya et al. 2014; Bahar et al. 2016) or no impact of the challenge (Vigors et al. 2016) in the challenged tissue (colon and ileum, respectively). As in these studies animals were older thus with a more developed immune system, comparison is difficult. However, Leonard et al. (2012) observed an interaction between maternal diet and LPS treatment in the ileum on TNF-α expression, using 26-days old piglets. This *ex vivo* system may thus not be the more appropriate and the dose of LPS and incubation time should be adapted; it is noteworthy that the variation between individuals cannot be excluded as a cause for inflammation failure.

Other *ex vivo* techniques also exist to mimic ETEC infection, including a porcine intestine organ culture (PIOC) that has been described by Aarhus University (Denmark) and allows quantifying the adhesion of pathogens, including ETEC, on the mucosal surface of the small intestine. This technique uses intestinal segment (10-15cm) that is filled with culture medium containing the pathogen. The segment is then sealed and incubated in culture medium for 1h, after which enumeration of pathogens is performed both on the inoculum and homogenised tissue to determine pathogen adhesion (Sugiharto et al. 2012). Another alternative would be infection trials, even though these cause biosecurity issues.

Because of the inefficiency of the LPS challenge, the mRNA levels of cytokines and tight junction proteins were measured directly in uncultured mucosa for the second animal experiment. Short-term results show no impact of the maternal RS diet on the expression of molecules involved in the inflammatory process (TNF-α, IL-6, NFκB, TGF-β, IFN-γ, IL-1β and IL-10), which is in agreement with Heim et al. (2015a) when feeding sows laminarin or fucoidan. However, on the long term, the maternal RS diet seemed to reduce IFN-γ together with increasing the expression of TNF-α, making the interpretation of data difficult for whether maternal RS supplementation could impact the inflammatory status of their progeny's gut.

Taken this together, the maternal supplementation with WB or RS did not seem to impact the immune ability of the piglets on the short term, even though effects on the long term were observed. However, the permeability of the membrane seemed to be

impacted positively by the fibre diets, but this may be verified with Ussing chambers or *in vivo* permeability studies.

High fat challenge

The aim of the long-term high fat study was to determine whether sows' diet could impact the ability of piglets to cope with the consequences of a high fat diet, namely appearance of an obese phenotype and global body inflammation.

A recurrent question that is raised concerning the pig as a model for human is whether this animal is suitable to mimic obesity and development of the metabolic syndrome, including type 2 diabetes. From the literature, the pig model is encouraged in comparison to rodent models due to the multiple similarities concerning diet, physiology and sedentary behaviour (Torres-Rovira et al. 2012). Moreover, the microbiota of pigs and humans share similarities and the main site of fermentation occurs in the colon, whereas fermentation occurs in the caecum for rodents (Heinritz et al. 2013). Comparatively to rodents, pigs also present the same number of beta cells in the pancreas relative to their bodyweight as humans (Renner et al. 2016). However, the site of lipogenesis differs between species, as it occurs in adipose tissue for pig and liver for human (Heinritz et al. 2013).

However, the use of the pig for description and cure of metabolic syndrome raises inevitably the limits of this model. Talking about the increase in fat deposition and appearance of insulin resistance that characterize the type 2 diabetes, conventional pigs may not be the most suitable model. Indeed, conventional pigs have been selected for leanness, resulting in barely any deposition of intramuscular or subcutaneaous fat even though fed ad libitum (Renner et al. 2016). Another problem with the use of conventional pigs is their fast growth that allows only the focus on infancy and adolescence in humans for studies. One way to study long term effect of a Western style diet is the use of minipigs, whose growth is rather limited and closer to humans', even though these animals are costly (Renner et al. 2016). Another solution would be the use of breeds that have not been selected for leanness, like Iberian pigs that are able to present most of the symptoms of metabolic syndrome, namely higher bodyweight and backfat, dyslipidaemia and insulin resistance after a high fat challenge (Torres-Rovira et al. 2012).

Even though getting obese pigs is very difficult, several parameters confirmed that pigs received a high amount of fat during the second animal experiment. Indeed, a higher backfat deposition and higher TG, TC and HDL levels in blood were observed for the HF animals, which are the first indicators of the development of an obese phenotype (Torres-Rovira et al. 2012). Dyslipidaemia had already been observed for conventional growing pigs (Puccinelli et al. 2015) and for Iberian pig (Torres-Rovira et al. 2012) after feeding the animals a high fat diet. Further analyses of RNAseq in the liver of pigs will give us better insights in the pathways that might affect liver cholesterol metabolism and biosynthesis. Microbiota composition was not affected by the diet either at the genus or phylum level and the ratio *Firmicutes*:*Bacteroidetes*

was not impacted. This is not surprising as Arnal et al. (2014) did not observe any microbiota changes related to the high fat diet either, using the same fat source at the same concentration in the diet. Gene expression of inflammatory cytokines or permeability in the colon of the animals was not affected, suggesting no chronical inflammation of the gut; however, we expect the inflammation to be detectable in the liver samples that will be analysed by RNAseq.

In addition, it would be worth to determine the expression of TLR4 in the intestine, which is the receptor for LPS and quantifying LPS blood concentration would be interesting as obesity is related to endotoxaemia (Boroni Moreira et al. 2012).

Thus, it is clear that the pig is a suitable model for studies concerning human metabolic disorders, even though the use of more rustic breeds or minipigs may be preferred. In this study, the level of incorporation of fat, the type of fat (saturated palm oil), the age of the pigs and the duration of the trial seemed appropriate. A longer duration could even have shown more impact on the appearance of the obese phenotype and microbiota, but the trial had to be shortened due to the high occurrence of umbilical hernias, mainly observed for the HF treated pigs.

As microbiota did not differ between diets but the SCFA did, it raises the question of whether sequencing is appropriate or sufficient. As most bacteria amplified during the sequencing process cannot be cultured, their mode of action is still unknown and does not give insights in their role for gut health. Thus, combining sequencing data with metatranscriptomics data may be the most suitable in order to determine whether a diet would effectively promote the growth of bacterial groups having a desired functionality, like butyrate or antimicrobial peptides production.

General conclusion and highlights of the thesis

Using maternal nutrition with dietary fibres may not be the best strategy to affect the microbiota of the progeny. However, using WB or RS in the diet of animals has the ability to shape their gut microbiota and alter the milk composition of the sows that can in turn impact several health-related parameters of their progeny. Thus, research might focus on the use of these prebiotics after birth in order to promote the establishment of a beneficial microbiota and efficient immune system as early in life as possible. Adding a sampling time point after weaning could also be very informative in terms of alterations in gut morphology and global inflammation.

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Leblois, J., Wavreille, J., Soyeurt, H., Dehareng, F., Grelet, C., Oswald, I.P., Li, B., Bindelle, J. & Everaert, N. Effects of a high wheat bran diet administrated to sows on performances and intestinal health parameters of the progeny. Submitted in Livestock Science (13-04-2018).

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